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(54) **HUMANIZED ANTI-CD40 MONOCLOINAL ANTIBODIES AND FRAGMENTS CAPABLE OF BLOCKING B CELL ACTIVATION**

HUMANISIERTE MONOKLONALE ANTI-CD40 ANTIKÖRPER UND FRAGMENTE ZUR HEMMUNG DER B-ZELLAKTIVIERUNG

ANTICORPS MONOCLONAUX HUMANISES DE TYPE ANTI-CD40 ET FRAGMENTS CAPABLES DE BLOQUER L'ACTIVATION DES LYMPHOCYTES B

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Description**FIELD OF THE INVENTION**

5 [0001] This invention relates to humanized anti-CD40 antibodies and antibody fragments. The antibodies may be used for preventing or treating antibody-mediated diseases such as IgE-mediated disease (allergies) and autoimmune diseases including systemic lupus erythematosus (SL), primary biliary cirrhosis (PC), idiopathic thrombocytopenic purpura (ITP), and rheumatoid arthritis (RA).

10 BACKGROUND OF THE INVENTION**I. B-Cell Activation**

15 [0002] B cells play an important role during the normal *in vivo* immune response. A foreign antigen will bind to surface immunoglobulins on specific B cells, triggering a chain of events including endocytosis, processing, presentation of processed peptides on MHC-class II molecules, and up-regulation of the B7 antigen on the B-cell surface. A specific T cell then binds to the B cell via T-cell receptor (TCR) recognition of processed antigen presented on the MHC-class II molecule. Stimulation through the TCR begins to activate the T cell and initiates T-cell cytokine production. Interaction between the CD28 antigen on T cells and the B7 antigen on B cells can provide a second signal further activating the

20 T cell, resulting in high level cytokine secretion.

Additionally, the CD40 ligand, which is not expressed on resting human T cells, is up-regulated on the T-cell surface when the above-mentioned signals are received. The B cell is then stimulated by The CD40 ligand through the CD40 antigen on the B-cell surface, and also by soluble cytokines, causing the B cell to mature into a plasma cell secreting high levels of soluble immunoglobulin.

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II. The EL4B5 Cell Line

30 [0003] A few years ago, Zubler *et al.*, J. Immunol. (1985) 134:3662, observed that a mutant subclone of the mouse thymoma EL-4 line, known as EL4B5, could strongly stimulate B cells of both murine and human origin to proliferate and differentiate into immunoglobulin-secreting plasma cells *in vitro*. This activation was found to be antigen-independent and not MHC restricted. For optimal stimulation of human B cells, the presence of supernatant from activated human T cells was needed, but a B-cell response also occurred when EL4B5 cells were preactivated with phorbol-12-myristate 13-acetate (PMA) or IL-1. Zubler *et al.*, Immunological Reviews (1987) 99:281; and Zhang *et al.*, J. Immunol. (1990) 144:2955. B-cell activation in this culture system is efficient -- limiting dilution experiments have shown

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that the majority of human B cells can be activated to proliferate and differentiate into antibody-secreting cells. Wen *et al.*, Eur. J. Immunol. (1987) 17:887.

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[0004] The mechanism by which these mutant EL-4 cells activate both murine and human B cells has not been elucidated previously. It is, however, clear that cell-cell contact is required for EL4B5-induced B-cell activation. First, B cells do not proliferate in the presence of supernatant from PMA-stimulated EL4B5 cells. Zubler *et al.* (1985) supra. Second, B cells do not proliferate when they are separated from PMA-treated EL4B5 cells by a semipermeable filter membrane. Zhang *et al.*, supra. Antibodies against mouse LFA-1, human LFA-1 or human LFA-3 and antibodies against mouse or human MHC class II molecules do not inhibit EL4B5-induced proliferation of human or murine B cells. Zubler *et al.* (1987) and Zhang *et al.*, supra.

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III. The CD40 Antigen, the CD40 Antigen Ligand, and Anti-CD40 Antibodies

50 [0005] The CD40 antigen is a glycoprotein expressed on the cell surface of B cells. During B-cell differentiation, the molecule is first expressed on pre-B cells and then disappears from the cell surface when the B cell becomes a plasma cell. Crosslinking of the CD40 molecules with anti-CD40 antibodies mediates a variety of effects on B cells. The CD40 antigen is known to be related to the human nerve growth factor (NGF) receptor and tumor necrosis factor-alpha (TNF- α) receptor, suggesting that CD40 is a receptor for a ligand with important functions in B-cell activation.

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[0006] A ligand for CD40 has been identified on the cell surface of activated T cells. Fenslow *et al.*, J. Immunol. (1992) 149:655; Lane *et al.*, Eur. J. Immunol. (1992) 22:2573; Noelle *et al.*, Proc. Natl. Acad. Sci. (USA) (1992) 89: 6550. cDNA cloning of the CD40 ligand revealed a molecule with characteristics of a type-II transmembrane glycoprotein with homology to TNF- α . Armitage *et al.*, Nature (1992) 357:80 and Spriggs *et al.*, J. Exp. Med. (1992) 176:1543. The extracellular domain of the CD40 ligand contains two arginine residues proximal to the transmembrane region, providing a potential proteolytic cleavage site that could give rise to a soluble form of the ligand. Expression of recombinant CD40 ligand has demonstrated that this molecule can stimulate the proliferation of purified B cells and, in com-

bination with IL-4, mediate the secretion of IgE. Armitage *et al.* and Spriggs *et al.*, *supra*. It has been reported that abnormalities in the gene for the CD40 ligand, resulting in the absence of a functional molecule on activated T cells, is responsible for the occurrence of X-linked hyper-IgM syndrome, a rare disorder characterised by the inability of these patients to produce normal levels of antibody isotypes other than IgM. Allen *et al.*, *Science* (1993) 259:990; and Korthäuer *et al.*, *Nature* (1993) 361:539.

[0007] Anti-CD40 antibodies may have a stimulatory effect on human B cells. Cross-linking of the CD40 molecule on the B-cell surface using known anti-CD40 antibodies mediates a variety of effects on B cells. Anti-CD40 monoclonal antibodies (mAbs) can induce intercellular adhesion, proliferation and, in combination with certain cytokines, maturation to antibody secreting cells. For example, known anti-CD40 mAbs have been shown to mimic the effects of T helper cells in B-cell activation. When presented on adherent cells expressing Fc_YRII, these antibodies induce B-cell proliferation. J. Bancereau *et al.*, *Science* (1989) 251:70. Moreover, the known anti-CD40 mAbs can replace the T helper signal for secretion of IgM, IgG and IgE in the presence of IL-4. H. Gascan *et al.*, *J. Immunol.* (1991) 147:8. Furthermore, known anti-CD40 mAbs can prevent programmed cell death (apoptosis) of B cells isolated from lymph nodes.

[0008] The anti-CD40 antibodies in the above art stimulate B cells but are incapable of inhibiting the B-cell response.

[0009] WO 94/01547 discloses a method for generation of antibodies to cell surface molecules, including anti-CD40 antibodies which inhibit B cell proliferation. Such antibodies are also disclosed in Kwekkeboom *et al.*, *Immunology*, 79: 439-440 (1993) which indicates such antibodies may inhibit activation of human B cells by murine EL 4B5 cells.

[0010] WO 93/11794 discloses methods and materials for preparation of modified antibody variable domains and therapeutic uses thereof. Padlan, *Molecular Immunology*, 28:489-498 (1991), discloses a procedure for reducing immunogenicity of antibody variable domains while preserving ligand binding properties.

SUMMARY OF THE INVENTION

[0011] The current invention is based on the discovery of anti-CD40 antibodies that do not stimulate the growth and differentiation of human B cells. In contrast, these antibodies can inhibit the human B-cell response at relatively low concentrations. Accordingly, these antibodies can be used to prevent or treat diseases or conditions that are mediated by antibodies produced by the human B-cell response. These antibodies also recognise novel epitopes on the CD40 antigen useful in modulating the B-cell response.

[0012] The present invention provides a humanized monoclonal antibody 5D12 obtainable from the deposited hybridoma cell line designated ATCC HB 11339, which antibody binds to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, said humanized monoclonal antibody 5D12 comprising the VH_x and VL_x amino acid sequences encoded by cDNA sequences which are depicted in Figures 12 and 13.

[0013] The invention further provides an antigen binding fragment of monoclonal antibody SD12 wherein said antibody is obtainable from the deposited hybridoma cell line designated ATCC HB 11339, said humanized monoclonal antibody 5D12 comprising the VH_x and VL_x amino acid sequences encoded by the cDNA sequences which are depicted in Figures 12 and 13. Such fragments may be Fab, F(ab')₂ or F_v fragments.

[0014] The invention also provides pharmaceutical compositions comprising a humanized monoclonal antibody or a humanized antigen binding fragment of the invention and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1A shows a schematic representation of the baculoviral transfer vector pAcC8 and the sequence of the multiple cloning site. Figure 1B shows a schematic representation of the generation of Sf9 cells which express human CD40 or B7 antigen.

[0016] Figure 2 shows the sequences of polymerase chain reaction primers used in the preparation of coding regions for human CD40 and human B7 antigens. These primers were constructed on the basis of the published complete DNA coding sequences for antigens B7 and CD40.

[0017] Figure 3 shows the results of ELISA assays examining the reaction of anti-CD40 monoclonal antibody S2C6 with Sf9 cells expressing CD40 and with Sf9 cells expressing B7.

[0018] Figure 4 shows the results of the fluorescent cell staining of EBV-transformed B-cell line ARC cells expressing CD40.

[0019] Figure 5A compares the ability of three new (5D12, 3C6 and 3A8) and one old (S2C6) anti-CD40 mAbs to co-stimulate anti-IgM induced human B-cell proliferation. Figure 5B repeats the experiment of Figure 5A in the presence of recombinant interleukin-2 (rIL-2).

[0020] Figure 6 shows the ability of three new anti-CD40 mAbs to inhibit human B-cell proliferation induced by co-stimulation with immobilized anti-IgM and anti-CD40 mAb S2C6.

[0021] Figure 7 shows the effect of three new anti-CD40 mAbs on EL4B5-induced human B-cell proliferation.

[0022] Figure 8 shows the effect of soluble CD40 (hCD40.h μ) on EL4B5-induced human B-cell proliferation;

[0023] Figures 9A and 9B show the effect of one new anti-CD40 mAb 5D12 on human T-cell induced immunoglobulin production of human B cells.

5 [0024] Figure 10 shows the DNA sequence for the murine heavy chain variable (VH_M) region of the 5D12 monoclonal antibody and the amino acid sequence encoded thereby, using conventional single letter designations. Figure 10 also labels VH_M at the first amino acid of each of the four framework regions (FR1, FR2, FR3 and FR4) and the three complementarity determining regions (CDR1, CDR2, CDR3) that are positioned therebetween in alternating fashion.

10 [0025] Figure 11 shows the DNA sequence for the murine light chain variable (VL_M) region of the 5D12 monoclonal antibody and the amino acid sequence encoded thereby, using conventional single letter designations. Figure 11 also labels VL_M the first amino acid of each of the four framework regions (FR1, FR2, FR3 and FR4) and the three complementarity determining regions (CDR1, CDR2 and CDR3) that are positioned therebetween in alternating fashion.

15 [0026] Figure 12 shows the DNA sequence for the humanized heavy chain variable (VH_x) region of 5D12 monoclonal antibody and the amino acid sequence encoded thereby, using conventional single letter designations. Figure 12 also labels VH_x the first amino acid of each of the four framework regions (FR1, FR2, FR3 and FR4) and the three complementarity determining regions (CDR1, CDR2 and CDR3) that are positioned therebetween in alternating fashion.

20 [0027] Figure 13 shows the DNA sequence for the humanized light chain variable (VL_x) region of the 5D12 monoclonal antibody and the amino acid sequence encoded thereby, using conventional single letter designations. Figure 13 also labels VL_x at the first amino acid residue of each of the four framework regions (FR1, FR2, FR3 and FR4) and the three complementarity determining regions (CDR1, CDR2 and CDR3) that are positioned therebetween in alternating fashion.

25 [0028] Figure 14 compares the binding to CD40 expressing cells by murine 5D12 monoclonal antibody, chimeric 5D12 Fab, chimeric VL, chimeric VH, humanized VL, humanized VH, and humanized 5D12 Fab, via FACScan flow cytometer curves, relative to each other and to FITC-conjugate as the control. Figure 14 shows that chimeric 5D12 Fab and humanized 5D12 Fab exhibit the same binding to CD40 expressing cells as does the intact 5D12 monoclonal antibody.

30 [0029] Figure 15 is a comparative plot of mean fluorescent intensity (MFI) versus Fab dilutions (1 = 16, 1=8, 1=4, 1=2 and 1=1) of chimeric 5D12 Fab (solid rectangle) and humanized 5D12 Fab (open ovals). To obtain the curve points, a CD40 expressing B cells (JY cell line) was incubated with 50 ng of intact 5D12 monoclonal antibody together with the diluted amounts of the expressed chimeric or humanized 5D12 Fab in 100 μ l for thirty minutes at 4°C, and the MFI was measured. The 1=1 dilution, which contained 70 ng chimeric 5D12 Fab or 80 ng humanized 5D12 Fab, was able to inhibit 50% of the intact murine 5D12 monoclonal antibody.

DETAILED DESCRIPTION OF THE INVENTION

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Definitions:

[0030] As used herein, the term "antibody" refers to polyclonal antibodies, monoclonal antibodies, humanized antibodies, single-chain antibodies, and fragments thereof such as F_{ab}, F_{(ab)2}, F_v, and other fragments, such as CDR fragments, which retain the antigen binding function of the parent antibody.

40 [0031] As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. The term encompasses whole immunoglobulins as well as fragments such as F_{ab}, F_{(ab)2}, F_v, and others, such as CDR fragments, which retain the antigen binding function of the antibody.

45 [0032] Monoclonal antibodies of any mammalian species can be used in this invention. In practice, however, the antibodies will typically be of rat or murine origin because of the availability of rat or murine cell lines for use in making the required hybrid cell lines or hybridomas to produce monoclonal antibodies.

50 [0033] As used herein, the term "humanized monoclonal antibodies" means that at least a portion of the exposed amino acids in the framework regions of the antibody (or fragment), which do not match with the corresponding amino acids in the most homologous human counterparts, are changed, such as by site directed mutagenesis of the DNA encoding the antibody. Because these exposed amino acids are on the surface of the molecule, this technique is called "resurfacing." Moreover, because the amino acids on the surface of the molecule are the ones most likely to give rise to an immune response, this resurfacing decreases the immunogenicity of the monoclonal antibody when administered to a species whose cell line was not used to generate the antibody, such as a human. The term "humanized monoclonal antibody" also includes chimeric antibody wherein the light and heavy variable regions of a monoclonal antibody generated by a hybridoma from a non-human cell line are each attached, via recombinant technology, to one human light chain constant region and at least one heavy chain constant region, respectively. The preparation of such chimeric (i.e., humanized) antibodies are disclosed by references incorporated herein by reference.

[0033] As used herein, the term "single chain antibodies" refers to antibodies prepared by determining the binding domains (both heavy and light chains) of a binding antibody, and supplying a linking moiety which permits preservation of the binding function. This forms, in essence, a radically abbreviated antibody, having only that part of the variable domain necessary for binding to the antigen. Determination and construction of single chain antibodies are described in U.S. Patent No. 4,946,779 to Ladner *et al.*

[0034] The term "CD40 antigen epitope" as used herein refers to molecule which is capable of immunoreactivity with the anti-CD40 monoclonal antibodies of this invention, excluding the CD40 antigen itself. CD40 antigen epitopes may comprise proteins, protein fragments, peptides, carbohydrates, lipids, and other molecules, but for the purposes of the present invention are most commonly proteins, short oligopeptides, oligopeptide mimics (*i.e.*, organic compounds which mimic the antibody binding properties of the CD40 antigen), or combinations thereof. Suitable oligopeptide mimics are described, *inter alia*, in WO 91/19735.

I. Antibody Preparation

[0035] Anti-CD40 monoclonal antibodies 5D12, 3A8 and 3C6 were prepared as described in Example 1 herein. Humanized anti-CD40 monoclonal antibody fragments were prepared as described in Example 8.

a) Polyclonal Sera

[0036] Polyclonal sera may be prepared by conventional methods. In general, a solution containing the CD40 antigen is first used to immunize a suitable animal, preferably a mouse, rat, rabbit, or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies. Immunization is generally performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 µg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vitro* immunization.

[0037] Polyclonal antisera are obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (*e.g.*, 1,000 x g for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

b) Monoclonal Antibodies

[0038] Monoclonal antibodies are prepared using the method of Kohler and Milstein, *Nature* (1975) 256:495-96, or a modification thereof, as are well known to the art. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) are removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen. B-cells expressing membrane-bound immunoglobulin specific for the antigen bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (*e.g.*, hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the desired immunizing cell-surface antigen (and which do not bind to unrelated antigens). The selected mAb-secreting hybridomas are then cultured either *in vitro* (*e.g.*, in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

[0039] If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ³²P and ¹²⁵I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. "Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific therefor. Other specific binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes. For example, ¹²⁵I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a mAb. Further, one may combine various labels for desired effect. For example, mAbs and avidin also require labels in the practice of this invention; thus, one might label a mAb with biotin, and detect its presence with

avidin labeled with ^{125}I , or with an anti-biotin mAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

CD40 Antigen Epitopes

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[0040] The CD40 antigen epitopes are molecules that are immunoreactive with anti-CD40 monoclonal antibodies whose binding to a human CD40 antigen located on the surface of a human B cell prevents the growth of differentiation of the B cell. That is, such epitopes compete with the binding of said antibodies to the CD40 antigen. Systematic techniques for identifying these epitopes are known in the art, as described by H.M. Geysen in U.S. Patent No. 4,708,871. Typically, these epitopes are short amino acid sequences. These sequences may be embedded in the sequence of longer peptides or proteins, as long as they are accessible.

[0041] The epitopes may be prepared by standard peptide synthesis techniques, such as solid-phase synthesis. Alternatively, the sequences may be incorporated into larger peptides or proteins by recombinant methods. This is most easily accomplished by preparing a DNA cassette which encodes the sequence of interest, and ligating the cassette into DNA encoding the protein to be modified at the appropriate site. The sequence DNA may be synthesized by standard synthetic techniques, or may be excised from the phage pIII gene using the appropriate restriction enzymes.

[0042] Epitopes identified herein may be prepared by simple solid-phase techniques. The minimum binding sequence may be determined systematically for each epitope by standard methods, for example, employing the method described by H.M. Geysen, U.S. Patent No. 4,708,871. Briefly, one may synthesize a set of overlapping oligopeptides derived

20 from the CD40 antigen bound to a solid phase array of pins, with a unique oligopeptide on each pin. The pins are arranged to match the format of a 96-well microtiter plate, permitting one to assay all pins simultaneously, e.g., for binding to an anti-CD40 monoclonal antibody. Using this method, one may readily determine the binding affinity for every possible subset of consecutive amino acids. Solid phase methods described in WO91/19785 may also be used to synthesise epitopes.

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Formulations and Methods of Administration

[0043] The antibodies of this invention are administered at a concentration that is therapeutically effective to prevent or treat antibody-mediated diseases such as allergies, SLE, PBC, ITP and RA. To accomplish this goal, the antibodies 30 may be formulated using a variety of acceptable excipients known in the art. Typically, the antibodies are administered by injection, either intravenously or intraperitoneally. Methods to accomplish this administration are known to those of ordinary skill in the art. It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

[0044] Before administration to patients, formants may be added to the antibodies. A liquid formulation is preferred. 35 For example, these formants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono-, di- or polysaccharides, or water soluble glucans. The saccharide or glucans can include fructose, dextrose, lactose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, alpha and beta cyclodextrin, soluble starch, hydroxethyl starch and carboxymethylcellulose, or mixtures thereof. Sucrose is most preferred. "Sugar alcohol" is defined as a C_4 to C_8 hydrocarbon having an -OH group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to amount used as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v% and 7.0 w/v%, more preferable between 2.0 and 6.0 w/v%. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; 40 however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the composition to minimize pH changes in the solution before lyophilization or after reconstitution.

45 Most any physiological buffer may be used, but citrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Most preferred is a citrate buffer. Preferably, the concentration is from 0.01 to 0.3 molar. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

[0045] Additionally, antibodies can be chemically modified by covalent conjugation to a polymer to increase their 50 circulating half-life, for example. Preferred polymers are disclosed in US patents 4,179,337; 4,495,285; and 4,609,546. Preferred polymers are polyoxylethylene polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: $\text{R}(\text{O}-\text{CH}_2-\text{CH}_2)_n\text{O}-\text{R}$ where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1,000 and 40,000, more preferably between 2,000 and 20,000, most

preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

5 [0046] Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), etc. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di- and triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf *et al.*, 1988 *J. Bio. Chem.*, 263:15064-15070, and a discussion of POG/IL-2 conjugates is found in U.S. Patent No. 4,766,106.

10 [0047] Another drug delivery system for increasing circulatory half-life is the liposome. Methods of preparing liposome delivery systems are discussed in Gabizon *et al.*, *Cancer Research* (1982) 42:4734; Cafiso, *Biochem. Biophys. Acta* (1981) 649:129; and Szoka, *Ann. Rev. Biophys. Eng.* (1980) 9:467. Other drug delivery systems are known in the art and are described in e.g., Poznansky *et al.*, *DRUG DELIVERY SYSTEMS* (R.L. Juliano, Ed., Oxford, N.Y. 1980), pp. 253-315; M.L. Poznansky, *Pharm. Revs.* (1984) 36:277.

15 [0048] After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

20 [0049] As stated above, the antibodies and compositions of this invention are used to treat human patients to prevent or treat antibody-mediate diseases such as allergies, SLE, PBC, ITP and RA. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are saline, Ringer's solution, dextrose solution, and Hanks' solution. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. A preferred vehicle is 5% dextrose in saline.

25 The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

30 [0050] The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that antibodies are given at a dose between 1 μ g/kg and 20 mg/kg, more preferably between 20 μ g/kg and 10 mg/kg, most preferably between 1 and 7 mg/kg. Preferably, it is given as a bolus dose, to increase circulating levels by 10-20 fold and for 4-6 hours after the bolus dose. Continuous infusion may also be used after the bolus dose.

35 If so, the antibodies may be infused at a dose between 5 and 20 μ g/kg/minute, more preferably between 7 and 15 μ g/kg/minute.

40 [0051] The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

Examples

Materials and Methods

45 Cell Lines

50 [0052] The mutant mouse thymoma EL-4 subclone EL4B5 was a gift of Dr. R.H. Zubler, Hôpital Cantonal Universitaire, Geneva. Mouse 3T6 transfected cells expressing hybrid molecules of the HR (high responder) allelic form of human F γ RIIa were a gift of Dr. P.A.M. Warmerdam, Department of Experimental Immunology, University Hospital Utrecht, Utrecht, The Netherlands. Warmerdam *et al.*, *J. Immunol.* (1991) 147:1338. Both cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM), supplemented with gentamicin (80 μ g/ml) and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, Utah). To avoid possible loss of B Cell activating capacity, every 4 to 8 weeks a new batch of EL4B5 cells was thawed. The cell lines were periodically tested for mycoplasma contamination by the use of a 3 H-labelled DNA probe for mycoplasma ribosomal RNA (GenProbe, San Diego, CA) and were free of mycoplasma during the course of the experiments.

Antibodies and hCD40. μ Fusion Protein

5 [0053] Anti-CD40 mAb 5D12, 3C6 and 3A8 were generated by immunizing mice with insect cells expressing recombinant human CD40 as shown in Example 1. Anti-(B7) mAb B7-24 was generated in a similar way by immunizing with insect cells expressing recombinant human B7. Anti-CD40 mAb S2C6 was a gift of Dr. S. Pauli (University of Stockholm, Sweden). Pauli et al., *J. Immunol.* (1989) 142:590. Anti-CD40 mAb G28.5 was donated by Dr. J.A. Ledbetter (Oncogen Corporation, Seattle, WA, USA). Clark et al., *PNAS(USA)* (1986) 83:4494. Control antibodies were: anti-(β -glucocerebrosidase) mAb 8E4 (IgG1), Barneveld et al., *Eur. J. Biochem.* (1983) 134:585, and myeloma immunoglobulins MOPC-21 (IgG1) and MOPC-141 (IgG2b) (Sigma, St. Louis, MO). All mAb were used as purified antibody preparations.

10 hCD40. μ fusion protein was a gift of Dr. P. Lane (Basel Institute for Immunology, Basel, Switzerland) and was used as a 5x concentrated supernatant of transfected J558L cells. Lane et al., *Eur. J. Immunol.* (1992) 22:2573.

Human B Lymphocytes

15 [0054] B lymphocytes were isolated from tonsils obtained from children undergoing tonsillectomies, essentially as described in De Groot et al., *Lymphokine Research* (1990) 9:321. Briefly, the tissue was dispersed with scalpel blades, phagocytic and NK cells were depleted by treatment with 5 mM L-leucine methyl ester and T cells were removed by one cycle of rosetting with sheep erythrocytes (SRBC) treated with 2-aminoethyl isothiouronium bromide. The purity of the resulting B lymphocyte preparation was checked with indirect immunofluorescent labelling with anti-(CD20) mAb B1 (Coulter Clone, Hialeah, FL) or anti-(CD3) mAb OKT3 (Ortho, Raritan, NJ) and a FITC-conjugated F(ab')₂ fragment of rabbit anti-(mouse Ig) (Zymed, San Francisco, CA), and FACS analysis. The B cell preparations contained (mean \pm SD of 6 isolations): 95 \pm 4% CD20-positive cells and 2 \pm 4% CD20-positive cells and 2 \pm 1% CD3-positive cells.

B-Cell Proliferation Assay

25 [0055] B cells (4×10^4 per well) were cultured in 200 μ l IMDM supplemented with 10% fetal calf serum in flat bottom 96-well microtiter plates. B cells were stimulated by addition of immobilized anti-(IgM) antibodies (Immunobeads*; 5 μ g/ml; BioRad*, Richmond, CA). Where indicated, 100 U/ml recombinant IL-2 was added. Varying concentrations of mAbs were added at the onset of the microcultures and proliferation was assessed at day 3 by measurement of the incorporation of [³H]-thymidine after eighteen hour pulsing.

Banchereau-Like-B-Cell Proliferation Assay

35 [0056] For testing the ability of anti-CD40 mAbs to stimulate B-cell proliferation in a culture system analogous to that described by Banchereau et al., *Science* (1991) 251:70, mouse 3T6 transfector cells expressing the HR allelic form of human Fc γ RII were used. B cells (2×10^4 per well) were cultured in flat-bottom microwells in the presence of 1×10^4 transfector cells (irradiated with 5000 Rad) in 200 μ l IMDM supplemented with 10% fetal calf serum and 200 U/ml recombinant IL-4. Before addition of the B cells, the 3T6 cells were allowed to adhere to the culture plastic for at least five hours. Anti-CD40 mAbs were added at concentrations varying from 15 mg/ml to 2000 ng/ml and proliferation of B cells were assessed by measurement of thymidine incorporation at day 7, upon eighteen hour pulsing with [³H]-thymidine.

B-Cell Activation Assay with EL4B5 Cells

45 [0057] B cells (1000 per well) were cultured together with irradiated (5000 Rad) EL4B5 cells (5×10^4 per well) in flat bottom microtiter plates in 200 μ l IMDM supplemented with 10% heat-inactivated fetal calf serum, 5 ng/ml phorbol-12-myristate 13-acetate (Sigma) and 5% human T-cell supernatant. MAbs were added at varying concentrations at the onset of the cultures and thymidine incorporation was assessed at day 6 after 28 hour pulsing with [³H]-thymidine. For the preparation of T-cell supernatant, purified T cells were cultured at a density of 10^6 /ml for 36 hours in the presence of 1 μ g/ml PHA and 10 ng/ml PMA. Wen et al. *supra*: T-cell supernatant was obtained by centrifugation of the cells and stored at -20°C. The effectiveness of T-cell supernatants in enhancing proliferation of human B cells in EL4B5-B cell cultures was tested and the most effective supernatants were pooled and used in the experiments.

Human T Cell Helper Assay for Antibody Production by Cells

55 [0058] 96-well tissue culture plates were coated with a 1:500 dilution of ascites fluid of anti-CD3 mAb CLB-T3/3 (CLB, Amsterdam, The Netherlands). As indicated, costimulatory mAbs were added: anti CD2 mAbs CLB-T11.1/1 and *Registered Trade Mark in one or more designated states.

CLB-T11.2/1 (CLB, Amsterdam, The Netherlands), both ascites 1:1000 and anti-CD28 mAb CLB-28/1 (CLB, Amsterdam, The Netherlands). Subsequently, tonsillar T cells (irradiated, 3000 Rad; 10⁵ per well), tonsillar B cells (10⁴ per well) and rIL-2 (20 U/ml) were added. The final volume of each cell culture was 200 µl. After eight days, cells were spun down, and cell-free supernatant was harvested. The concentrations of human IgM and IgG in (diluted) samples were estimated by ELISA as described below.

5 ELISA Assay for Immunoglobulin Quantification

[0059] The concentrations of human IgM and IgG were estimated by ELISA. 96-well ELISA plates were coated with 4 µg/ml mouse anti-human IgG mAb MH 16-01 (CLB, Amsterdam, The Netherlands) or with 1.2 µg/ml mouse anti-human IgM mAb 4102 (Tago, Burlingame, CA) in 0.05 M carbonate buffer (pH = 9.6), by incubation for 16 h at 4°C. Plates were washed three times with PBS-0.05 % Tween-20 (PBS-Tween)* and saturated with BSA for one hour. After two washes, the plates were incubated for one hour at 37°C with different dilutions of the test samples. After three washes, bound Ig was detected by incubation for 1 hour at 37°C with 1 µg/ml horseradish peroxidase-labeled mouse anti-human IgG mAb MH 16-01 (CLB) or mouse anti-human IgM mAb MH 15-01 (CLB). Plates were washed four times and bound peroxidase activity was revealed by the addition of o-phenylenediamine as a substrate. Human standard serum (H00, CLB) was used to establish a standard curve for each assay.

20 Flow Cytometric Assay

[0060] ARC cells (10⁶ cells/sample) were incubated in 100 µl primary antibody (10 µg/ml in PBS-BSA or Hanks' balanced salt solution (HBSS) supplemented with 1% BSA and 0.05% sodium azide) for twenty minutes at 4°C. After three washes with PBS-BSA or HBSS-BSA, the cells were incubated in 100 µl FITC-labeled F(ab')₂ fragments of goat anti-(mouse IgG) antibodies (Jackson, West Grove, PA) for twenty minutes at 4°C. After three washes with PBS-BSA or HBSS-BSA and one wash with PBS, the cells were resuspended in 0.5 ml PBS. Analyses were performed with a FACSCAN V (Becton-Dickinson, San Jose, CA).

[0061] Alternatively, EL4B5 cells were harvested before and at different time points during culture in medium containing PMA (5ng/ml) and human T-cell supernatant (5%). Cells were incubated for thirty minutes with 10 µl supernatant of transfected cells containing hCD40.hp diluted in 100 µl Hank's Balanced Salt Solution supplemented with 0.05% sodium azide (4°C). This was followed by incubation with FITC-conjugated F_{(ab')2} fragments of rabbit anti-(human IgM) (Central Laboratory of the Netherlands, Blood Transfusion Service, Amsterdam, The Netherlands). As a control, cells were incubated with the FITC-conjugate only. For analysis, a FACScan-4 cytofluorometer (Becton-Dickinson) was used. Non-vital cells were excluded from analysis by the use of propidium iodide.

35 c) Humanized Monoclonal Antibodies

[0062] The monoclonal antibodies described above, which are capable of blocking the CD40-CD40 ligand interaction, are a useful therapeutic tool in treating autoimmune diseases. However, because many of said monoclonal antibodies are derived from a non-human (e.g., murine) cell line, the antibodies may be recognized over time as foreign by the human recipient and an immune response may be mounted which would neutralize subsequent administrations of the monoclonal antibodies. Because the immunogenicity of a foreign protein is largely determined by the nature of the surface, it was decided that the immunogenicity of any monoclonal antibodies that were derived from non-human cell lines could be reduced or abolished if an effective number of the exposed (i.e., surface) amino acids in the framework regions of the antibody were replaced with those amino acids usually found in human antibodies.

[0063] Techniques for humanizing monoclonal antibodies are well known in the art. Moreover, the techniques for humanizing a monoclonal antibody which are disclosed in the following publications.

- 1) Rodwell, "Engineering Monoclonal Antibodies," *Nature*, **342**:99-100 (1989);
- 2) Reichmann, *et al.*, "Reshaping Human Antibodies For Therapy," *Nature*, **332**:323-327 (1988);
- 3) Waldman, Thomas, "Monoclonal Antibodies in Diagnosis and Therapy," *Science*, **252**:1657-1662 (1991);
- 4) Oi, *et al.*, "Chimeric Antibodies," *BioTechniques*, **4**(3):214:221 (1986);
- 5) Liu, *et al.*, "Production Of A Mouse-Human Chimeric Monoclonal Antibody To CD20 With Patent Fc-Dependent Biologic Activity," *J. Immunol.*, **139**:3521-3526 (1987);

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6) Beidler, *et al.*, "Cloning and High Level Expression of A Chimeric Antibody With Specificity For Human Carcinoembryonic Antigen," *J. Immunol.*, **141**:4053-4060 (1986);

5 7) Jones, *et al.*, "Replacing The Complementarity-Determining Regions In A Human Antibody With Those From A Mouse," *Nature*, **321**:522-525 (1986);

8) Wood, *et al.*, "The Synthesis And In Vivo Assembly of Functional Antibodies In Yeast," *Nature*, **314**:446-449 (1985);

10 9) U.S. Patent 4,816,567 (Cabilly, *et al.*) "Recombinant Immunoglobulin Preparations";

10) Morrison, "Transfectedomas Provide Novel Chimeric Antibodies," *Science* **229**:1202-1207 (1985);

15 11) Better, *et al.*, "Escherichia coli Secretion of an Active Chimeric Antibody Fragment," *Science*, **240**:1041-1043 (1988);

12) Boulian, G.L., *et al.*, "Production of Functional Chimaeric Mouse/Human Antibody," *Nature* **312**:643-646 (1984); and

20 13) Heuberger, *et al.*, "A Hapten-specific Chimaeric IgE antibody With Human Physiological Effector Function," *Nature* **314**: 268-270 (March, 1985).

[0064] For example, to humanize a monoclonal antibody, the variable heavy (VH) chain and the variable light (VL) chain of the monoclonal antibody to be humanized are cloned, sequenced, compared to a series of the most homologous human sequences and then the DNA sequence encoding the monoclonal antibody is modified by site directed mutagenesis. In particular, messenger RNA encoding the monoclonal antibody to be humanized is obtained from the hybridoma producing that antibody. To obtain mRNA encoding the monoclonal antibody, the hybridoma cells are washed twice with phosphate buffered saline and lysed with quanidium thiocyanate in the presence of 0.7M 2-mercaptoethanol. The cell lysate is layered on a discontinuous CsCl gradient and centrifuged for about sixteen hours at 25 26,000 rpm in a Beckman* centrifuge having an SW28 rotor. The RNA is recovered by dissolving the resulting pellet in diethyl pyrocarbonate-treated H₂O (DEPC-treated H₂O). The RNA is precipitated with ethanol once resuspended in DEPC-treated H₂O, and stored at -70°C.

[0065] Amplification of the cDNA encoding the heavy and light chain variable regions of the mAb is accomplished by polymerase chain reaction (PCR) using a set of degenerate primers with restriction sites for cloning. The typical primer set consists of eight 5' primers for the variable region of the heavy chain (VH) that are combined with one universal 3' primer located in the constant region of the heavy chain (CH), and five 5' primers for the variable region of the light chain (VL) that are combined with one universal 3' primer located in the constant region of the light chain (CL). Optionally, a determination is made that one of the primer sets is best suited for the amplification of the VH and VL regions. Thereafter, multiple PCR runs are performed using a low cycle number to avoid incorporation of PCR mistakes. The PCR products obtained after about 24 amplification cycles are subcloned in the polylinker of a sequencing vector. Several (e.g., six) independent clones are analyzed for both VH and VL. This results in a consensus sequence for the cDNA and the deduced protein sequence of the VH and VL regions, respectively of the monoclonal antibody. The deduced protein sequence is overlayed with the location of the framework regions (FR) and the complementarity determining regions (CDR) which form the antigen-binding site of the antibody.

[0066] To produce a humanized antibody with the least likelihood of generating an immune response in a human, the deduced protein sequences of the VH and VL regions are used to search different data bases for human antibody sequence with the best homology to the monoclonal antibody (or fragment if only a fragment is to be humanized.) The results are tabulated for the VH and VL regions. Several (e.g., three) of the best matching human antibody sequences are used to determine which of the non-human amino acids in the exposed framework should be changed to a human 45 amino acid in order to obtain a humanized version of the monoclonal antibody.

[0067] PCR primers are designed to change the selected exposed residues from non-human to human. Those skilled in the art recognize that the encoding number of mutagenic primers that are designed depends upon the number of mutagenic sites to be introduced into the VH and VL regions. For example, to introduce a total of seven non-adjacent mutagenic sites in both the VH and VL regions, a total of eight primers are designed for the mutagenesis of the VH 55 region, and likewise eight primers are designed for the VL region. cDNA encoding the non-human sequence is used as template for the PCR reactions. Both the humanized variable heavy region (VH_x) and the humanized variable light region (VL_x) region are constructed in three consecutive PCR steps. After the final PCR step, the PCR construct is

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subcloned in the polylinker of a sequencing vector.

5 [0068] The VH_x and VL_x subclones are transferred using standard techniques from the sequence vectors to expression vectors that already coded part of the human constant heavy (CH_H) region and the complete human constant light (CL_H) region, thereby producing an expression vectors encoding VH_xCH_H and VL_xCL_H , respectively. The expression vectors coding VH_xCH_H and VL_xCL_H are cotransfected into Sf9 insect cells, whereupon the transfected insect cells secrete the antibody as a **humanized** monoclonal antibody fragment.

10 [0069] Alternatively, to prepare a chimeric monoclonal antibody from a non-human monoclonal antibody, the variable regions of the original non-human (e.g., mouse) heavy and light chains (VH_M and VL_M) of the monoclonal antibody, as described above were transferred respectively into the expression above that already coded the CH_H and CL_H regions of a human Fab fragment, thereby producing expression vectors encoding VH_MCH_H and VL_MCL_H into Sf9 insect cells, the transfected insect cells secrete the antibody as a human/mouse chimeric monoclonal antibody fragment.

Example 1

15 Making Monoclonal Antibodies to B7 and CD40

A. PCR Cloning of CD40 and B7

20 [0070] RNA was isolated from a population of EBV-transformed human spleen cells, essentially as described by Chirgwin *et al.*, *Biochemistry* (1979) 17:5294. In brief, the cells were washed twice with phosphate buffered sale (PBS) and lysed in 5 M guanidinium thiocyanate in the presence of 0.7 M 2-mercaptoethanol. The cell lysate was layered on a discontinuous CsCl gradient (Chirgwin *et al.*) and centrifuged for sixteen hours at 26,000 rpm in a Beckman SW28 rotor. The RNA was recovered by dissolving the pellet in DEPC-treated H_2O . The RNA was precipitated with ethanol once, resuspended in DEPC-treated H_2O , and stored at -70°C.

25 [0071] Total RNA (10 μ g/reaction) was converted to cDNA using random hexamer priming in 50 μ l reaction buffer containing 500 units MLV-RT (Bethesda Research Laboratories, Bethesda, MD), 5 μ M random hexamer (Pharmacia, Piscataway, NJ), 1 mM MgCl₂ and 0.1 mg/ml BSA (bovine serum albumin). After incubation at 37°C for one hour, the samples were boiled for three minutes and stored at -70°C. The DNA encoding the CD40 and B7 molecules was 30 generated by PCR using primers which contained sequences having homology to known CD40 and B7 sequence, where the primers also encoded restriction sites useful for cloning (Figure 2). These primers were based on the published cDNA coding sequences for B7 and CD40. Freeman *et al.*, *J. Immunol.* (1989) 143:1714, and Stamenkovic *et al.*, *EMBO J.* (1989) 8:1403. All primers start with a C-G clamp at the 5' end followed by a restriction site for cloning (shown in bold, Figure 2). The underlined sequences in the backward primers, for the cloning of the soluble forms of 35 B7 and CD40, represents an epitope recognized by a monoclonal antibody used for affinity purification. The numbers in brackets represent the location of the primers relative to the published cDNAs for CD40 and B7.

40 [0072] For PCR amplification, 1 μ l of cDNA was mixed with 1 μ l (10 picomoles) of a forward primer, 1 μ l (10 picomoles) of a backward primer, and 47 μ l of PCR mix. The PCR mix consisted of 1.25 units Taq polymerase (Perkin-Elmer/ Cetus, Norwalk, CT), dNTP mix (0.2 mM each), 10 mM tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl₂ and 0.1 mg/ml BSA. The 50 μ l of PCR mixture was overlaid with 70 μ l mineral oil and subjected to 25 cycles of amplification in a Perkin-Elmer/Cetus thermocycler (denaturation at 95°C for thirty seconds, primer annealing at 55°C for thirty seconds and extension at 72°C for 1.5 minutes). PCR products were obtained after 25 amplification cycles.

45 [0073] The amplification products were digested with *BgIII* and *KpnI* (Figure 1B) and isolated by size-fractionation. Before expression in baculovirus, the DNA sequence of each fragment was confirmed by sequencing analysis to prevent the introduction of PCR-induced mutations. The baculovirus transfer vector pAcC8 was also digested with *BgIII* and *KpnI* (Figure 1B).

50 [0074] The amplified fragments were ligated to the linear pAcC8 vector (ratio of insert to vector was 3:1). The ligation products were transformed into bacterial strain DH5 α (Gibco/BRL, Gaithersburg MD) and recombinant pAcC8 vectors were selected on the basis of ampicillin resistance. Recombinant plasmids were isolated from bacterial clones (Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor, NY: Cold Spring Harbor Laboratories, 1982; Ausubel *et al.*, *Current Protocols in Molecular Biology* (Media, PA: John Wiley and Sons)) and the presence of the insert of interest verified using polymerase chain reactions (see above). Large scale plasmid preparation was performed by standard procedures (Ausubel *et al.*; Maniatis *et al.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor, NY: Cold Spring Harbor Laboratories), 1989).

55 B. Baculovirus Expression of Human CD40 and B7

[0075] Sequences encoding human CD40 and human B7 were recombined into the *Autographa californica* baculo-

virus (AcNPV) using the transfer vectors pAcCD40 (encoding the full-length CD40 molecule), pAcCD40-ED/Glu (encoding the extracellular domain of CD40), pAcB7 (encoding the full-length B7 molecule) and pCcB7-ED/Glu (encoding the cellular domain of the B7 molecule).

5 [0076] The plasmids were cotransfected with wild-type baculoviral DNA (2-10 pfu) (AcNPV; Summers *et al.*, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experimental Station Bulletin No. 1555 (1987) into Sf9 (*Spodoptera frugiperda*) cells at a density of 10^6 cells/ml (Summers *et al.*). Recombinant baculovirus-infected Sf9 cells were identified and clonally purified (Summers *et al.*).

[0077] For cell surface expression of recombinant proteins, the cells were harvested after forty-eights hours of culture.

10 **C. Sf9 Insect Cell ELISA**

[0078] Sf9 insect cells infected with recombinant virus were cultured for 48 hours in 24-well plates. After removal of the tissue culture medium, the plates were incubated for 45 minutes at room temperature (RT) with 0.25 ml of antibody in PBS with 1% BSA (PBS-BSA). After three washes with PBS-BSA, the plates were incubated for 35 minutes at RT with 250 μ l of a 1/250 dilution of goat anti-(mouse total Ig) immunoglobulins conjugated to horseradish peroxidase (Zymed, South San Francisco, CA) in PBS-BSA. Unbound peroxidase activity was removed by washing five times with PBS-BSA. Bound peroxidase activity was revealed by the addition of an assay mixture prepared by diluting 0.5 ml of 2 mg/ml 3,3',5,5'-tetramethylbenzidine in ethanol to 10 ml with 10 mM sodium acetate, 10 mM EDTA buffer (pH 5.0) and adding 0.03% (v/v) H₂O₂. The reaction was stopped after ten minutes by adding 100 μ l of 1 M H₂SO₄.

15 [0079] The above-described ELISA assays performed on live Sf9 cells gave the following results. Figure 3 presents the data for live Sf9 cells infected with pAcB7 and pAcCD40 which were cultured for 48 hours in 24-well plates. The antibodies used in the ELISA to generate Figure 3 were: S2C6 (anti-CD40, open bars) and no primary antibody (hatched bars).

20 **D. Host Animal Immunization**

[0080] Female BALB/c mice were injected intraperitoneally at day 0 and day 14 with 5×10^6 Sf9 cells infected with AcCD40 virus, AcB7 virus or AcCD3 virus (control virus). At day 21, 100 μ l of serum was obtained to test for the presence of specific antibodies. After a rest period of at least two weeks, the mice received a final injection with 5×10^6 cells infected with AcCD40 or AcB7 virus. Three days after this last injection, the spleen cells were used for cell fusion.

25 **E. Generation of Hybridoma Clones**

30 [0081] Splenocytes from immunized BALB/c mice were fused with SP2/0 murine myeloma cells at a ratio of 10:1 using 50% polyethylene glycol as previously described by de Boer *et al.*, J. Immunol. Meth. (1988)113:143. The fused cells were resuspended in complete IMDM medium supplemented with hypoxanthine (0.1 mM), thymidine (0.016 mM) and 0.5 ng/ml hIL-6 (Genzyme, Cambridge, MA). The fused cells were then distributed between the wells of 96-well tissue culture plates, so that each well contained 1 growing hybridoma on average.

35 [0082] After 10-14 days, the supernatants of the hybridoma populations were screened for specific antibody production. For the screening of specific antibody production by the hybridoma clones, the supernatants of twelve wells were pooled and used for fluorescent cell staining of EBV-transformed B cells as described for the FACS Assay above. Subsequently, the supernatants of the positive pools were tested individually. Positive hybridoma cells were cloned three times by limiting dilution in IMDM/FBS containing 0.5 ng/ml hIL-6. Three hybridomas producing anti-CD40 antibodies are labelled 5D12, 3A8 and 3C6. The data is presented in Figure 4, which shows that a soluble form of CD40, but not of B7 can block the binding of the anti-CD40 mAb 5D12 to CD40 expressing EBV-transformed B cells. Figure 4 shows fluorescent cell staining of ARC EBV-transformed B cells with 5D12 is the presence and absence of soluble B7 and soluble CD40. 5D12 and the soluble B7, soluble CD40, or controls were preincubated at RT for 20 minutes before addition to the ARC cells. Figure 4A shows staining with 5D12 (dotted line) or second antibody only (solid line). 40 Figure 4B shows staining with 5D12 alone (dotted line) or preincubated with soluble B7 (solid line). Figure 4C shows staining with 5D12 alone (dotted line) or preincubated with soluble CD40.

45 **Example 2**

50 **55 Costimulation of B-Cell Proliferation Using Anti-CD40 mAbs**

[0083] Four hybridomas producing monoclonal antibodies against human CD40 were generated in Example 1. These mAbs were shown to bind to a similar proportion of tonsillar B cells as anti-CD40 mAb G28.5 does. de Boer *et al.* J.

Immunol. Methods (1992) 152:15. Three of these monoclonal antibodies (5D12, 3A8 and 3C6) which were of the IgG2b subclass, were tested for their ability to deliver activation signals to human B cells in the B-cell proliferation assay described above.

5 [0084] Human tonsillar B cells (4×10^4 per well) were cultured in 200 μ l in microwells in the presence of anti-IgM coupled to Sepharose beads (5 μ g/ml) (Figure 5A) or in the presence of anti-IgM plus rIL-2 (100 U/ml) (Figure 5B). Varying concentrations of the anti-CD40 mAbs S2C6, 5D12, 3C6 or 3A8 were added and [3 H]thymidine incorporation was measured at day 3 after 18 hours of pulsing. Data presented in Figure 5A are means derived from experiments with B-cell preparations from three different donors with duplicate incubations. Data of Figure 5B are means of duplicate incubations from one experiment out of two with comparable results.

10 [0085] None of the novel anti-CD40 mAbs was able to significantly costimulate human B-cell proliferation in the presence of immobilized anti-IgM or in the presence of immobilized anti-IgM and IL-2. In contrast, anti-CD40 mAb S2C6 costimulated human B-cell proliferation in a concentration dependent fashion.

15 **Example 3**

15 **Induction of B-Cell Proliferation Using Anti-CD40 mAbs**

20 [0086] The mAbs tested in Example 2 were tested for their ability to induce proliferation of human B cells in the Banchereau-like Assay described above, *i.e.*, by presenting the anti-CD40 mAb on adherent cells expressing Fc γ RII. As antibody presenting cells, mouse 3T6 transfectant cells expressing the HR allelic form of human Fc γ RII were used. It was observed that anti-CD40 mAb S2C6 together with IL-4 induced substantial proliferation of tonsillar human B cells in this system, as assessed by measurement of [3 H]thymidine incorporation. Anti-CD50 mAbs 5D12, 3C6 or 3A8, however, did not induce proliferation of human B cells in this culture system (data not shown).

25 **Example 4**

25 **Inhibition of S2C6 Stimulated B-Cell Proliferation Using Anti-CD40 mAbs**

30 [0087] The mAbs were also tested for their ability to inhibit the costimulation of human B-cell proliferation by anti-CD40 mAb S2C6 using the B-cell proliferation Assay described above. Human tonsillar B cells (4×10^4 per well) were cultured in 200 μ l in microwells in the presence of anti-IgM coupled to Sepharose* beads (5 μ g/ml) and anti-CD40 mAb S2C6 (1.25 μ g/ml). Varying concentrations of anti-CD40 mAbs 5D12, 3C6 or 3A8 were added and [3 H]thymidine incorporation was assessed after three days. As a control anti-(glucocerebrosidase) mAb 8E4 was added in similar concentrations. Barneveld *et al.* Eur. J. Biochem. (1983) 134:585. Data are means \pm S.D. derived from experiments with B cells from two different donors with duplicate incubations.

35 [0088] It was found that each of the anti-CD40 mAbs 5D12, 3A8 and 3C6 could inhibit the costimulation of anti-IgM induced human B-cell proliferation by mAb S2C6 (Figure 6). In contrast, no significant inhibition was seen with equivalent amounts of non-relevant mAb 8E4, directed to β -glucocerebrosidase. Barneveld *et al.*, *supra*. Thus, it was concluded that these anti-CD40 mAbs do not deliver stimulatory signals to the proliferation of human B cells, but, conversely, can inhibit stimulatory signals exerted by triggering anti-CD40 with another mAb. Therefore, these mAbs were considered to be excellent tools to investigate whether signaling via CD40 plays a role in the stimulation of human B-cell proliferation by EL4B5 cells.

40 **Example 5**

45 **Effects of Anti-CD40 mAbs on EL4B5-Induced Human B-Cell Proliferation**

50 [0089] The effects of anti-CD40 mAbs on EL4B5-induced human B-cell proliferation was tested using the B-cell Activation Assay described above. Human tonsillar B cells (1000 per well) were cultured together with irradiated EL4B5 cells (50,000 per well) in the presence of 5% supernatant of activated human T cells and 5 ng/ml PMA. Anti-CD40 mAbs 5D12, 3C6 or 3A8 were added in varying concentrations. As a control, mAb MOPC-141 (IgG2b) was added. After six days of culture, [3 H]thymidine incorporation was assessed.

55 [0090] Figure 7 shows that addition of anti-CD40 mAbs 5D12, 3C6 or 3A8 resulted in a concentration-dependent inhibition of human B-cell proliferation. Data are means \pm S.D. derived from experiments with B cells from four different donors with duplicate incubations. [3 H]-thymidine incorporation values found for incubations without mAb were (means \pm S.D.) 10460 \pm 1843 cpm, 6982 \pm 1729 cpm, 4362 \pm 1020 cpm and 1543 \pm 3190 in the four different experiments, respectively. [3 H]-thymidine incorporation in B cells alone amounted to 40 \pm 5 cpm and in irradiated EL4B5 cells alone

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31 ± 15 cpm.

[0091] Very potent inhibition occurred. At concentrations as low as 10 ng/ml each, the three anti-CD40 mAbs 5D12, 3C and 3A8 inhibited human B-cell proliferation completely. Halfmaximal inhibition was found at about 1 ng/ml. In contrast, isotype matched IgG2b mouse myeloma protein MOPC-141 had no significant effect on [³H]-thymidine incorporation. Similarly inhibition was observed when [³H]-thymidine incorporation was assessed at day 4 of the culture instead of day 6, thus excluding the possibility that the observed effect was due to a change in the kinetics of the proliferation under influence of the anti-CD40 mAb (data not shown).

[0092] For comparison, the influence of a few mAb directed against other B-cell surface structures was investigated. Neither anti-CD20 mAb B1 or anti-B7 mAb B7-24 (the latter mAb was generated by a procedure similar to that used for generating the anti-CD40 mAb used in Figure 7) in concentrations similar to those used in the experiments with the anti-CD40 mAb, had any effect on EL4B5-induced human B-cell proliferation (data not shown). Therefore, it may be concluded that the inhibitory effect of anti-CD40 mAb on EL4B5-induced B-cell proliferation is not due to masking of the B-cell surface.

15 **Example 6**

Effects of hCD40.h_μ on EL4B5-Induced Human B-Cell Proliferation

[0093] In order to investigate whether EL4B5 cells expressed a membrane structure which binds CD40, a fusion protein consisting of the extracellular domain of CD40 and human IgM constant domains CH₂, CH₃ and CH₄ (hCD40.h_μ) was used for flow fluorocytometric analysis. Lane et al., *supra*. Non-activated EL4B5 cells did not bind the fusion protein. However, upon culturing EL4B5 cells together with PMA (5 ng/ml) and 5% human T-cell supernatant, which are the conditions needed for activation of human B cells, a low binding of hCD40.h_μ was found (data not shown). This small shift in fluorescence was found consistently in three independent experiments. The minimal activation period needed for induction of the CD40 binding was 24 hours. To determine whether binding of hCD40.h_μ to the EL4B5 cells would inhibit EL4B5-induced human B-cell proliferation like anti-CD40 mAb did, the fusion protein was titrated into cocultures of EL4B5 cells with human B cells using the B-cell Activation Assay described above. Figure 8 shows that the fusion protein did indeed inhibit [³H]-thymidine incorporation in a concentration-dependent manner and, like the anti-CD40 mAb used in the experiments shown in Figure 7, was able to inhibit B-cell proliferation induced by the EL4B5 cells completely.

Example 7

Effects of Anti-CD40 mAbs on Human t-Cell-Induced Antibody Production by Human B-Cells

[0094] The antibodies were also tested for their capacity to inhibit immunoglobulin production by B cells, stimulated in a contact-dependent manner with activated T cells using the T-cell helper assay described above. Human tonsillar B cells (10⁴/well) were cultured together with irradiated purified T cells (3000 rad, 10⁵ /well) in 96-well plates, coated with anti-CD3 mAb and with or without different mAbs to costimulate the T cells. After 8 days of culture the supernatants were harvested for the determination of immunoglobulin production by the B cells. Immunoglobulin production by the B cells was assessed by the ELISA assay described above. Anti-CD40 mAb 5D12 was added in varying concentrations from the onset of the cultures. As a control, mAb 5D12 was added in varying concentrations from the onset of the cultures. As a control, mAb MOPC-141 was added. Figure 9A shows that when T cells were stimulated with immobilized anti-CD3 mAb and costimulated with soluble anti-CD2 and anti-CD28 mAbs, addition of anti-CD40 mAb 5D12 resulted in a concentration dependent inhibition of IgG production by human B cells. IgM production by the B cells was inhibited to the same extent. Similar results were obtained with the anti-CD40 mAbs 3C6 and 3A8 and with the hCD40.h_μ fusion protein.

[0095] The anti-CD40 mAbs of this invention exhibited very potent inhibitory. At concentrations as low as approximately 30 ng/ml, each of the three anti-CD40 mAbs gave a 50% of maximal inhibition. In contrast, the isotype-matched IgG_{2b} mouse myeloma protein MOPC-141 had no effect on the immunoglobulin production.

[0096] The inhibitory effect by the three anti-CD40 mAbs was not specific for the manner of activation of the T cells providing the CD40 ligand helper activity. Figure 9B shows that under all the T-cell stimulation conditions (anti-CD3 alone; anti-CD3 + anti-CD2; anti-CD3 + anti-CD28; and anti-CD3 + anti-CD2 + anti-CD28), the addition of the anti-CD40 mAb 5D12 results in strong inhibition of immunoglobulin production by the human B cells. The inhibition is comparable to the amount of inhibition with the hCD40.h_μ fusion protein, known to completely block the CD40-CD40 ligand interaction. The percentage of inhibition varied from 40 to 70% depending on the T-cell activation conditions. In contrast, the isotype-matched IgG_{2b} mouse myeloma protein MOPC-141, or human IgM (as control for the hCD40.h_μ fusion protein) had no effect on immunoglobulin production by the human B cells.

Example 8

Humanizing Anti-CD40 Monoclonal Antibodies

5 [0097] To humanize the Fab fragment of the 5D12 monoclonal antibody, the variable heavy (VH) chain and the variable light (VL) chain of 5D12 monoclonal antibody were first cloned and then sequenced. To obtain messenger RNA encoding for the 5D12 monoclonal antibody, the hybridoma cells that produce the 5D12 monoclonal antibody were washed with phosphate buffered saline, lysed in 5M guanidinium thiocyanate in the presence of 2-mercaptoethanol (see Example 1A). The lysate was then incubated with oligo-dT beads to bind the mRNA. To amplify the variable (VH and VL) regions of 5D12, the mRNA was used in RT-PCR employing degenerate primers for the IgG variable light (VL) chain or variable heavy (VH) chain. RT-PCR is a modification of the PCR technique that permits the amplification of sequences found in RNA target polynucleotides by first generating a cDNA product which is then amplified by PCR. The RT-PCR technique is well known in the art see for example Myers *et al.*, *Biochemistry* 30:7661-7666 (1991) and U.S. Patent Nos. 5,310,652 and 5,407,800.

10 15 [0098] The PCR products were cloned using TA-cloning in a sequencing plasmid. The DNA sequence of several independent clones encoding VH and VL were determined. From these DNA sequences, a consensus amino acid sequence for both the VH and VL regions of the monoclonal antibody 5D12 were deduced. Figures 10 and 11 show the cDNA and the deduced protein sequence of the VH and VL regions, respectively of monoclonal antibody 5D12. Also indicated on Figures 10 and 11 are the locations of the four framework regions (as FR1, FR2, FR3 and FR4) and 20 the three complementarity determining regions (as CDR1, CDR2, and CDR3) which are positioned therebetween.

25 [0099] The deduced amino acid sequences of the VH and VL regions were used to search different databases for human antibody sequences with the best homology to 5D12. The results of this search comparing ten homologous sequences (H1 to H10) relative to the deduced sequences for the VH and VL regions of 5D12 were used to decide which mouse residues to change. In particular, it was decided that nine (9) residues be changed in the VH region and four (4) residues be changed in the VL region.

30 [0100] The next step in the humanization process of 5D12 was to design mutagenesis primers to change the indicated residues from mouse to human. From the plasmids containing the VH or VL mouse variable regions, single stranded DNA was made by infection with R408 helper phage, followed by annealing of the mutagenesis primers. Double stranded DNA was made by T4 DNA polymerase and T4 DNA ligase together with dNTPs. Several clones were sequenced 35 to find a clone containing all of the introduced mutations. Figures 12 and 13 show the cDNA and the deduced amino acid sequence of the humanized VH and VL regions, respectively, now designated as VH_x and VL_x, respectively.

40 [0101] To obtain humanized Fab fragments of SD12, the two humanized variable regions (VH_x and VL_x) were each cloned in a baculovirus (AcNPV) expression plasmid (see Example 1B) that included part of the constant region of the human IgG, heavy chain (CH_H) and the complete human constant light (CL_H) region respectively, thereby producing expression vectors encoding VH_xCH_H and VL_xCL_H, respectively. The two expression vectors from above, encoding VH_xCH_H and VL_xCL_H were cotransfected with wildtype AcNPV baculovirus into Sf9 insect cells (see Example 1B) followed by a few rounds of amplification to obtain high titer recombinant virus stocks. To express the humanized 5D12 Fab fragment, the Sf9 cells were infected with the humanized light and heavy chain recombinant virus stocks for four days. The supernatants and lysates were analyzed for humanized 5D12 Fab expression using Western blotting and FACS analysis (Figure 14). In the resulting 5D12 Fab fragment, the light chain had 223 amino acid residues, and the heavy chain had 239 amino acid residues.

45 [0102] Similarly, a human/mouse chimeric 5D12 Fab fragment was produced. In particular, the cDNA encoding the two mouse variable regions (VH_M and VL_M) of 5D12, as obtained above, were each cloned in a baculovirus (AcNPV) expression plasmid (see Example 1B) that includes a portion of the constant region of the human IgG heavy chain (CH_H) and the complete constant light (CL_H) region respectively, thereby producing expression vectors encoding VH_MCH_H and VL_MCL_H, respectively. The two expression vectors encoding VH_MCH_H and VL_MCL_H were cotransfected with wildtype AcNPV baculovirus into Sf9 insect cells (see Example 1B) followed by a few rounds of amplification to obtain high titer recombinant virus stocks. To express the chimeric human/mouse 5D12 Fab fragment, the Sf9 cells were infected with the chimeric heavy and light chain recombinant virus stocks for four days. The supernatants and lysates 50 were analyzed for chimeric 5D12 Fab expression using Western blotting and FACS analysis (Figure 14).

Western Blot

55 [0103] A Western blot of an SDS-PAGE 10% gel, which was run under non-reducing conditions, was stained with 4-chloro-naphthol. Lanes 1 and 5, which were samples of culture supernatants from Sf9 cells infected with humanized 5D12 Fab fragment and chimeric 5D12 Fab fragment, showed comparable migration across from the 44 kD marker. Lanes 3 and 7, which were samples of culture supernatants from Sf9 cells infected with humanized light chain (VL_xCL_H) and chimeric light chain (VL_MCL_H) also showed comparable migration that reflected a molecular weight substantially

below the 32 kD marker.

FACS Binding Analysis

5 [0104] Using the few cytometric assay techniques disclosed therein, the binding of the anti-human CD40 5D12 monoclonal antibody and the various expressed Fab fragments was determined relative to the B-cell line JY, which expresses high levels of CD40. Cells (500,000/pellet) were incubated with 1 µg 5D12 in a volume of 100 µl or with 100 µl SF9 culture supernatant (which contained the VL, VH or Fab fragments) for thirty min. at 4°C. After washing with PBS, the cells were incubated with FITC labelled antibodies directed either against murine Ig or against the Fab fragments. Subsequently, the cells were washed and fixed in 0.5% paraformaldehyde in PBS. The cells were analyzed on a FACScan flow cytometer (Becton-Dickson). The results, which are shown in Figure 14, reflect that with the "humanized" 5D12 Fab fragment and the "chimeric" 5D12 Fab fragment bound to the CD40 antigen on the B cells comparable to the intact 5D12 murine monoclonal antibody, and that the chimeric VL and HL fragments, and the humanized VL and HL fragments exhibited binding that was indistinguishable from that of the non-binding control (i.e., FITC-conjugate only).

FACS Competition Experiment

20 [0105] JY cells (250,000/pellet) expressing CD40 were incubated with 50 ng intact 5D12 together with different amounts of the expressed chimeric or humanized 5D12 Fab in 100 µl for thirty minutes at 4°C. The 1:1 dilution contained 70 ng chimeric 5D12 Fab or 80 ng humanized 5D12 Fab (Fab concentration was determined with a sandwich ELISA using goat anti-human IgG as coating, human IgG Fab fragment from Jackson as standard and phosphatase labelled goat anti-human IgG F(ab')₂ as detection antibody). After washing with PBS, the cells were incubated with FITC labelled anti-murine Ig, which does not crossreact with the expressed Fab fragments, followed by fixation in 0.5% paraformaldehyde. Cells were analyzed on a FACScan flow cytometer (Becton-Dickinson). The mean fluorescence intensity (MFI) was plotted against the amount of added Fab fragment expressed as a dilution, and is shown in Figure 15. In the above FACS competition experiment, the chimeric 5D12 fragment and the humanized 5D12 Fab showed the same competitive binding characteristics relative to the parent murine 5D12 monoclonal antibody. See Figure 15. About 70 - 80 ng of the Fab fragment (humanized or chimeric) was found to inhibit 50% binding of the whole murine 5D12 antibody (50 ng). See Figure 15.

Deposition of Cultures

25 [0106] The hybridomas used in the above examples, to illustrate the method of the present invention were deposited in and accepted by the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA, under the terms of the Budapest Treaty.

Hybridoma	Deposit Date	Accession No.
B7-24	May 6, 1993	HB 11341
3C6	May 6, 1993	HB 11340
5D12	May 6, 1993	HB 11339
3A8		HB 12024

SEQUENCE LISTING

[0107]

(1) GENERAL INFORMATION:

50

(i) APPLICANT: CHIRON CORPORATION

55

(ii) TITLE OF INVENTION: Humanized Anti-CD40 Monoclonal Antibodies and Fragments Capable of Blocking B Cell Activation

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CHIRON CORPORATION Intellectual Property - R440
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5 (C) CITY: Emeryville
(D) STATE: California
(E) COUNTRY: United States of America
(F) ZIP: 94662-8097

10 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
15 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

20 (A) APPLICATION NUMBER: Unassigned
(B) FILING DATE: Even date herewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

25 (A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

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(C) TELEX: n/a

40 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 339 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

50 (ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 1..339

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

EP 0 896 585 B1

5	CAG GTG AAG CTC GAG TCT GGA CCT GGC CTG GTG GCA CCC TCA CAG Gln Val Lys Leu Glu Glu Ser Gly Pro Gly Leu Val Ala Pro Ser Gin 1 5 10 15	48
10	AGC CTG TCC ATC ACA TGC ACT GTC TCT GGG TTC TCA TTA TCC AGA TAT Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Leu Ser Arg Tyr 20 25 30	96
15	AGT GTA TAC TGG GTT CGC CAG CCT CCA GGA AAG GGT CTG GAG TGG CTG Ser Val Tyr Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Leu 35 40 45	144
20	GGA ATG ATG TGG GGT GGT GGA TCC ACA GAC TAT AAT TCA GCT CTC AAA Gly Met Met Trp Gly Gly Ser Thr Asp Tyr Asn Ser Ala Leu Lys 50 55 60	192
25	TCC AGA CTG AGC ATC AGC AAG GAC ACC TCG AAG AGC CAG GTC TTC TTA Ser Arg Leu Ser Ile Ser Lys Asp Thr Ser Lys Ser Gln Val Phe Leu 65 70 75 80	240
30	AAA ATG AAC AGT CTG CAA ACT GAT GAC ACA GCC ATG TAC TAC TGT GTC Lys Met Asn Ser Leu Gln Thr Asp Asp Thr Ala Met Tyr Tyr Cys Val 85 90 95	288
35	AGA ACC GAT GGG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC Arg Thr Asp Gly Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser 100 105 110	336
40	TCA Ser	339

(2) INFORMATION FOR SEQ ID NO:2:

35

- (A) LENGTH: 113 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Met Met Trp Gly Gly Ser Thr Asp Tyr Asn Ser Ala Leu Lys
50 55 60

5 Ser Arg Leu Ser Ile Ser Lys Asp Thr Ser Lys Ser Gln Val Phe Leu
65 70 75 80

10 Lys Met Asn Ser Leu Gln Thr Asp Asp Thr Ala Met Tyr Tyr Cys Val
85 90 95

15 Arg Thr Asp Gly Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser
100 105 110

15 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: Protein

(ix) FEATURE:

30 (A) NAME/KEY: CDS
(B) LOCATION: 1..336

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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5	GAG CTC CAG CTG ACC CAG TCT CCA CTC TCC CTG CCT GTC AGT CTT GGA Glu Leu Gln Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Ser Leu Gly 115 120 125	48
10	GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA AAC AGT Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val Asn Ser 130 135 140 145	96
15	AAT GGA AAC ACC TAT TTA CAT TGG TAC CTG CAG AAG CCA GGC CAG TCT Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser 150 155 160	144
20	CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro 165 170 175	192
25	GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC Asp Arg Phe Ser Gly Ser Gly Ser Gly-Thr Asp-Phe Thr-Leu-Lys Ile 180 185 190	240
30	AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser 195 200 205	288
	ACA CAT GTT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys 210 215 220 225	336

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 112 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Glu Leu Gln Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Ser Leu Gly
 1 5 10 15

5 Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val Asn Ser
 20 25 30

10 Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser
 35 40 45

15 Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
 50 55 60

20 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65 70 75 80

25 Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
 85 90 95

30 Thr His Val Pro Trp Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys
 100 105 110

(2) INFORMATION FOR SEQ ID NO:5:

25 (i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 339 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: Protein

40 (ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 1..339

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAG GTG CAA CTC GTG GAG TCT GGA CCT GGC CTG GTG AAA CCC TCA CAG 48
 Gln Val Gln Leu Val Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln
 45 115 120 125

AGC CTG TCC ATC ACA TGC ACT GTC TCT GGG TTC TCA TTA TCC AGA TAT 96
 Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Leu Ser Arg Tyr
 50 130 135 140

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5 AGT GTA TAC TGG GTT CGC CAG CCT CCA GGA AAG GGT CTG GAG TGG CTG 144
 Ser Val Tyr Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Leu
 145 150 155 160

10 GGA ATG ATG TGG GGT GGT GGA TCC ACA GAC TAT AAT TCA GCT CTC AAA 192
 Gly Met Met Trp Gly Gly Ser Thr Asp Tyr Asn Ser Ala Leu Lys
 165 170 175

15 TCC AGA CTG ACC ATC AGC AAG GAC ACC TCG AAG AAC CAG GTC TTC TTA 240
 Ser Arg Leu Thr Ile Ser Lys Asp Thr Ser Lys Asn Gln Val Phe Leu
 180 185 190

20 AAA ATG AAC AGT CTG CGA GCT GAG GAC ACA GCC ATG TAC TAC TGT GTC 288
 Lys Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Met Tyr Tyr Cys Val
 195 200 205

25 AGA ACC GAT GGG GAC TAC TGG GGT CAA GGA ACC ACC GTC ACC GTC TCC 336
 Arg Thr Asp Gly Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser
 210 215 220

TCA 339
 Ser
 225

(2) INFORMATION FOR SEQ ID NO:6:

30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 113 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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	Gln	Val	Gln	Leu	Val	Glu	Ser	Gly	Pro	Gly	Leu	Val	Lys	Pro	Ser	Gln
	1		5		10		15									
5	Ser	Leu	Ser	Ile	Thr	Cys	Thr	Val	Ser	Gly	Phe	Ser	Leu	Ser	Arg	Tyr
	20			25		30										
10	Ser	Val	Tyr	Trp	Val	Arg	Gln	Pro	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Leu
	35		40		45											
15	Gly	Met	Met	Trp	Gly	Gly	Ser	Thr	Asp	Tyr	Asn	Ser	Ala	Leu	Lys	
	50		55		60											
20	Ser	Arg	Leu	Thr	Ile	Ser	Lys	Asp	Thr	Ser	Lys	Asn	Gln	Val	Phe	Leu
	65		70		75		80									
	Lys	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Met	Tyr	Tyr	Cys	Val
	85		90		95											
25	Arg	Thr	Asp	Gly	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser
	100		105		110											

Ser

(2) INFORMATION FOR SEQ ID NO:7:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: Protein
(ix) FEATURE:40 (A) NAME/KEY: CDS
(B) LOCATION: 1..336

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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	GAC CTC CAG CTG ACC CAG CAG TCT CCA CTC TCC CTG CCT GTC AGT CTT GGA	48		
	Asp Leu Gln Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Ser Leu Gly			
115	120	125		
5	GAT CGA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA AAC AGT	96		
	Asp Arg Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val Asn Ser			
130	135	140	145	
10	AAT GGA AAC ACC TAT TTA CAT TGG TAC CTG CAG AAG CCA GGC CAG TCT	144		
	Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser			
	150	155	160	
15	CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA	192		
	Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro			
	165	170	175	
20	GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC	240		
	Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile			
	180	185	190	
25	AGC AGA GTG GAG GCT GAG GAT GTG GGA GTT TAT TAC TGC TCT CAA AGT	288		
	Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Ser Gln Ser			
	195	200	205	
	ACA CAT GTT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA	336		
	Thr His Val Pro Trp Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys			
	210	215	220	225

30 (2) INFORMATION FOR SEQ ID NO:8:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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Asp Leu Gln Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Ser Leu Gly
 1 5 10 15

5 Asp Arg Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val Asn Ser
 20 25 30

Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser
 10 35 40 45

Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
 50 55 60

15 Asp Arg Phe Ser Gly Thr Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Ser Gln Ser
 20 85 90 95

Thr His Val Pro Trp Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys
 100 105 110

25 **Claims**

1. Humanized monoclonal antibody 5D12 obtainable from the deposited hybridoma cell line designated ATCC HB 11339, which antibody binds to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, said humanized monoclonal antibody 5D12 comprising the VH_x and VL_x amino acid sequences encoded by the cDNA sequences which are depicted in Figures 12 and 13.
2. A humanized antigen binding fragment of monoclonal antibody 5D12 wherein said antibody is obtainable from the deposited hybridoma cell line designated ATCC HB 11339, said humanized monoclonal antibody 5D12 comprising the VH_x and VL_x amino acid sequences encoded by the cDNA sequences which are depicted in Figures 12 and 13.
3. The fragment of claim 2 which is Fab, F(ab')₂ or F_v.
4. The fragment of claim 3, which is Fab.
5. A pharmaceutical composition comprising a humanized monoclonal antibody or a humanized antigen binding fragment of any one of claims 1, 2, 3 or 4 and a pharmaceutically acceptable carrier.

45 **Patentansprüche**

1. Humanisierter monoklonaler Antikörper 5D12 erhältlich aus der hinterlegten Hybridoma-Zelllinie mit der Bezeichnung ATCC HB 11339, der an ein menschliches CD40-Antigen, welches sich auf der Oberfläche einer humanen B-Zelle befindet, bindet, wobei die Bindung des Antikörpers an das CD40-Antigen das Wachstum oder die Differenzierung der B-Zelle verhindert, wobei der humanisierte monoklonale Antikörper 5D12 die VH_x und VL_x-Aminosäuresequenzen umfasst, welche durch die cDNA-Sequenzen codiert werden, die in den Figuren 12 und 13 beschrieben sind.
2. Humanisiertes Antigen-bindendes Fragment des monoklonalen Antikörpers 5D12, worin der Antikörper erhältlich ist aus der hinterlegten Hybridoma-Zelllinie mit der Bezeichnung ATCC HB 11339, wobei der humanisierte monoklonale Antikörper 5D12 die VH_x und VL_x-Aminosäuresequenzen umfasst, die durch die cDNA-Sequenzen codiert werden, welche in den Figuren 12 und 13 beschrieben sind.

3. Fragment nach Anspruch 2, nämlich Fab, F(ab') oder F_v.
4. Fragment nach Anspruch 3, nämlich Fab.
5. Pharmazeutische Zusammensetzung umfassend einen humanisierten monoklonalen Antikörper oder ein humanisiertes Antigen-bindendes Fragment nach einem der Ansprüche 1, 2, 3 oder 4 und einen pharmazeutisch annehmbaren Träger.

10 **Revendications**

1. Anticorps monoclonal humanisé 5D12 pouvant être obtenu à partir de la lignée cellulaire d'hybridome déposée désignée par ATCC HB 11339, cet anticorps se liant à un antigène CD40 humain situé sur la surface d'une cellule B humaine, la liaison de l'anticorps à l'antigène CD40 empêchant la croissance ou la différenciation de la cellule B, ledit anticorps monoclonal humanisé 5D12 comprenant les séquences d'acides aminés VH_x et VL_x codées par les séquences d'ADNc qui sont présentées aux figures 12 et 13.
2. Fragment de liaison à un antigène humanisé de l'anticorps monoclonal 5D12, ledit anticorps pouvant être obtenu à partir de la lignée cellulaire d'hybridome déposée désignée par ATCC HB 11339, ledit anticorps monoclonal humanisé 5D12 comprenant les séquences d'acides aminés VH_x et VL_x codées par les séquences d'ADNc qui sont présentées aux figures 12 et 13.
3. Fragment de la revendication 2, qui est Fab, F(ab')₂ or F_v.
4. Fragment de la revendication 3, qui est Fab.
5. Composition pharmaceutique comprenant un anticorps monoclonal humanisé ou un fragment de liaison à un antigène humanisé de l'une quelconque des revendications 1, 2, 3 ou 4 et un véhicule pharmaceutiquement acceptable.

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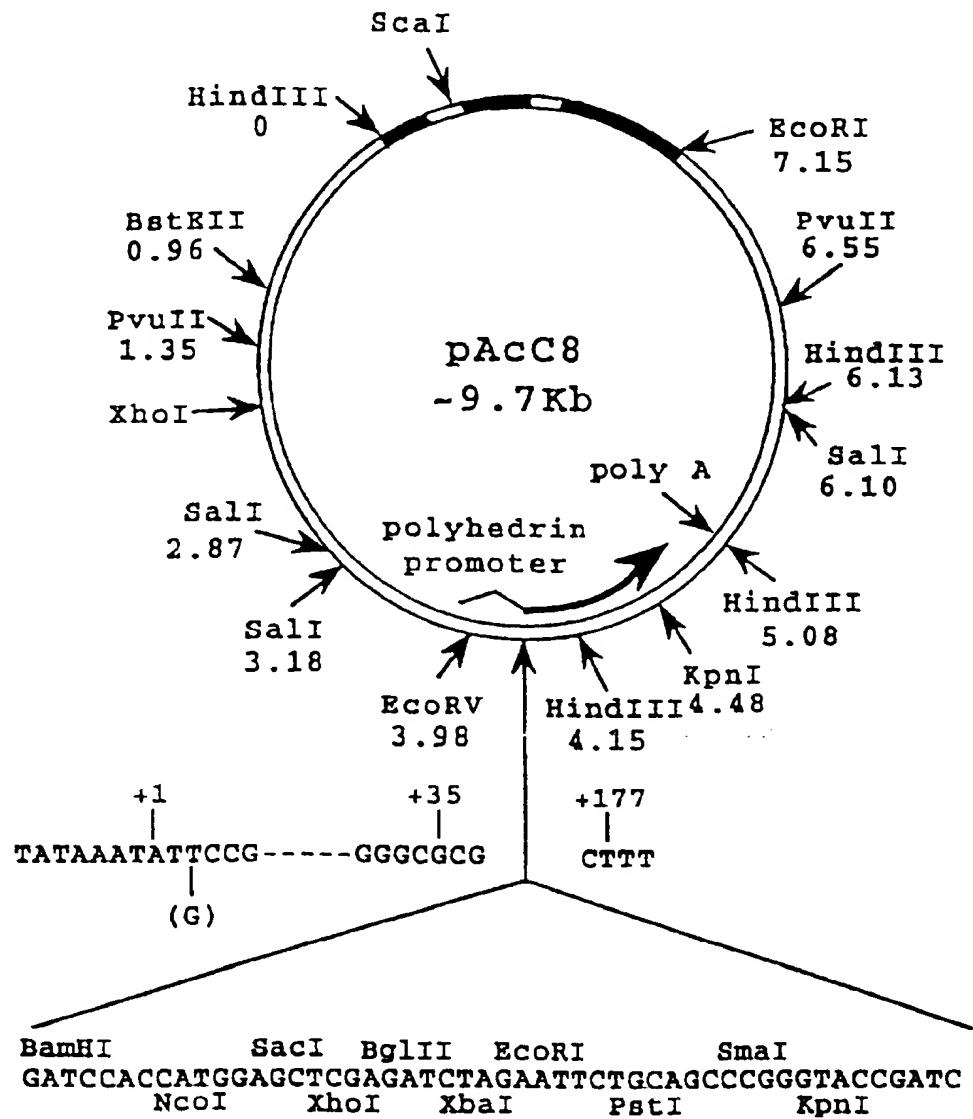


FIG. IA

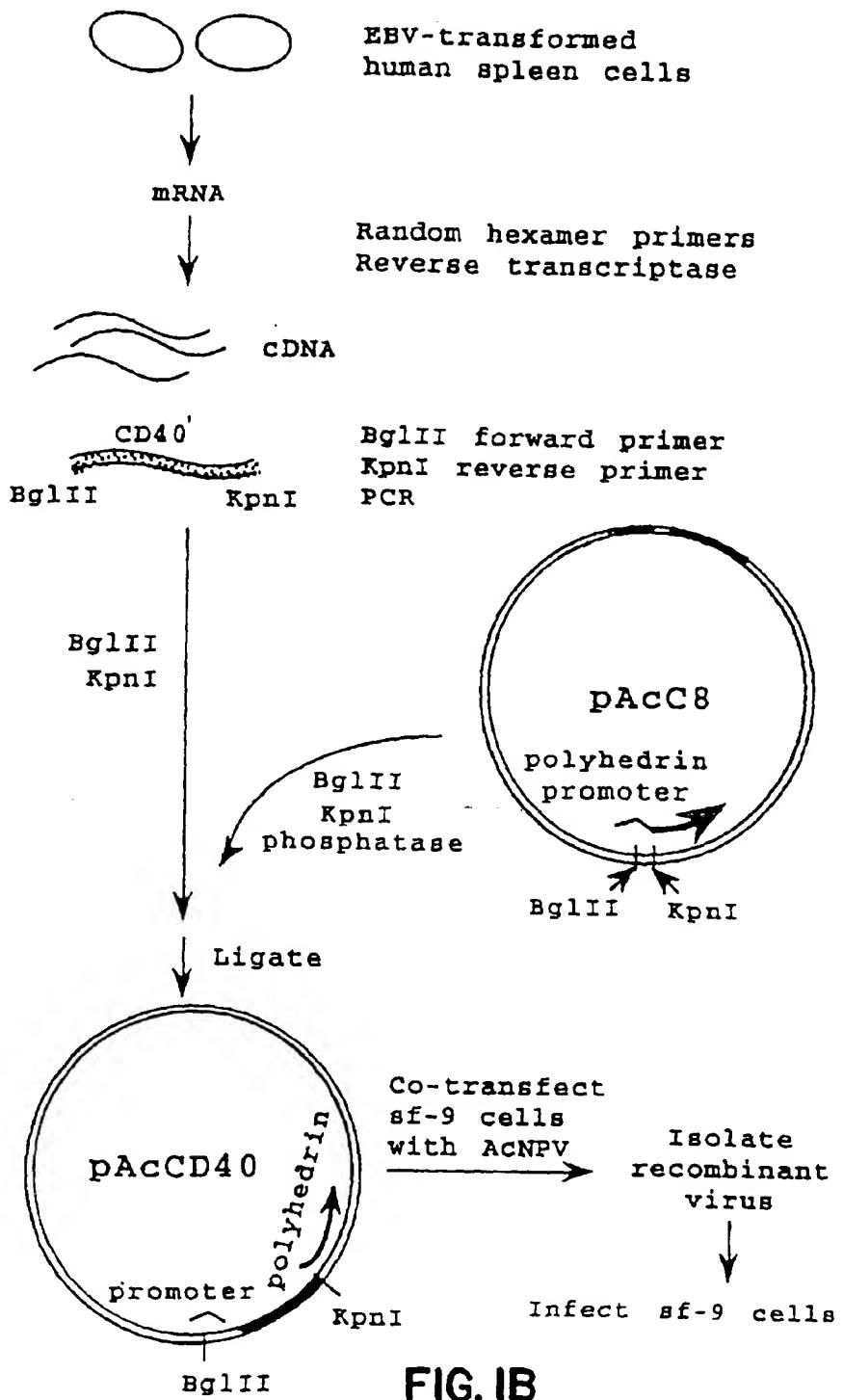


FIG. 1B

Full length B7:

Forward MR67 5'-GCG CTGGAG CATCTGAACCATGGGCC-3' (307-324)

Backward MR68 5'-GGC GGTACC TTGCTTCTGGGACACTG-3' (1182-1199)

Soluble B7:

Forward MR67 5'-GCG CTGGAG CATCTGAAGCCATGGGCC-3' (307-324)

Backward MR145 5'-GGC GGTACC TTACTCCATGGGAGATGATTCGTTATCAGGAAAATGCTGTTG-3' (1022-1042)

Full length CD40:

Forward MR108 5'-GGGT AGATCT GGTCTCACCTCGCCATGGTTCG-3' (34-55)

Backward MR112 5'-GGGT GGTACC CCACACTCTGGGGGGAGGCC-3' (882-905)

Soluble CD40:

Forward MR108 5'-GGGT AGATCT GGTCTCACCTCGCCATGGTTCG-3' (34-55)

Backward MR150 5'-GGGT GGTACC TTACTCCATGGGAGATGATTCGTTGCTGCTGC-3' (575-596)

FIG. 2

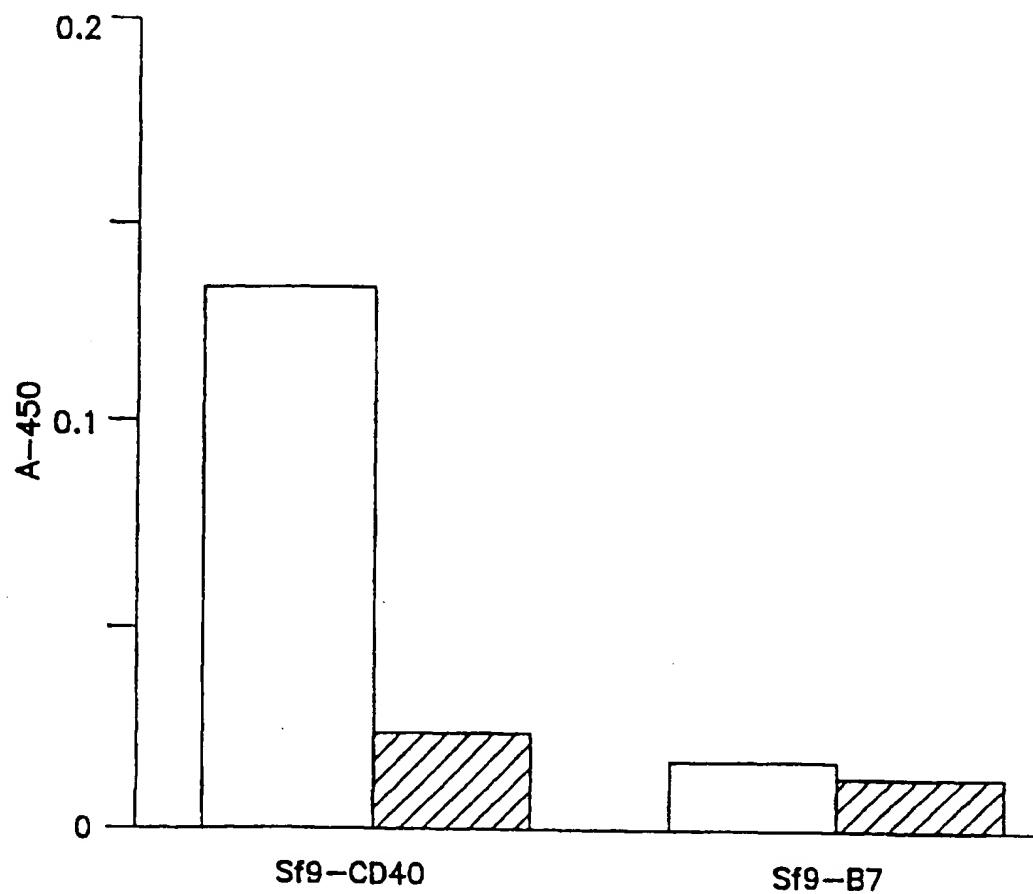


FIG. 3

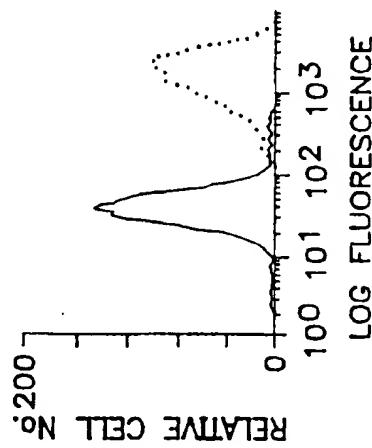


FIG. 4C

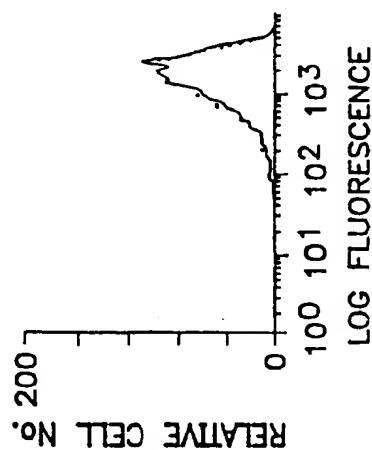


FIG. 4B

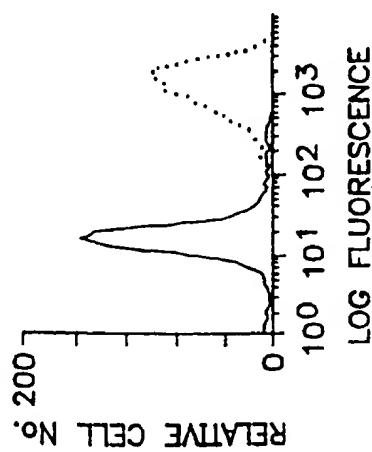
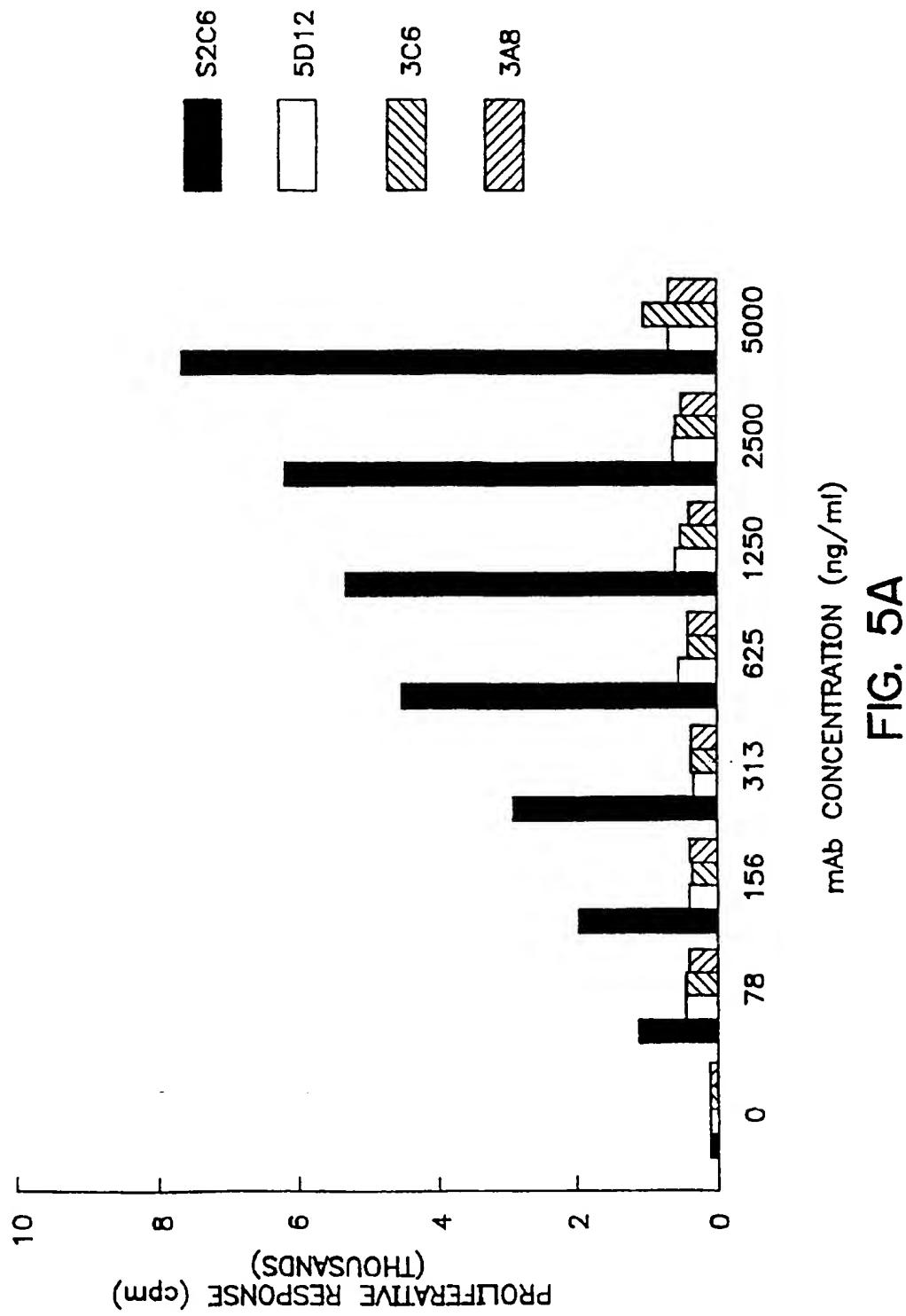
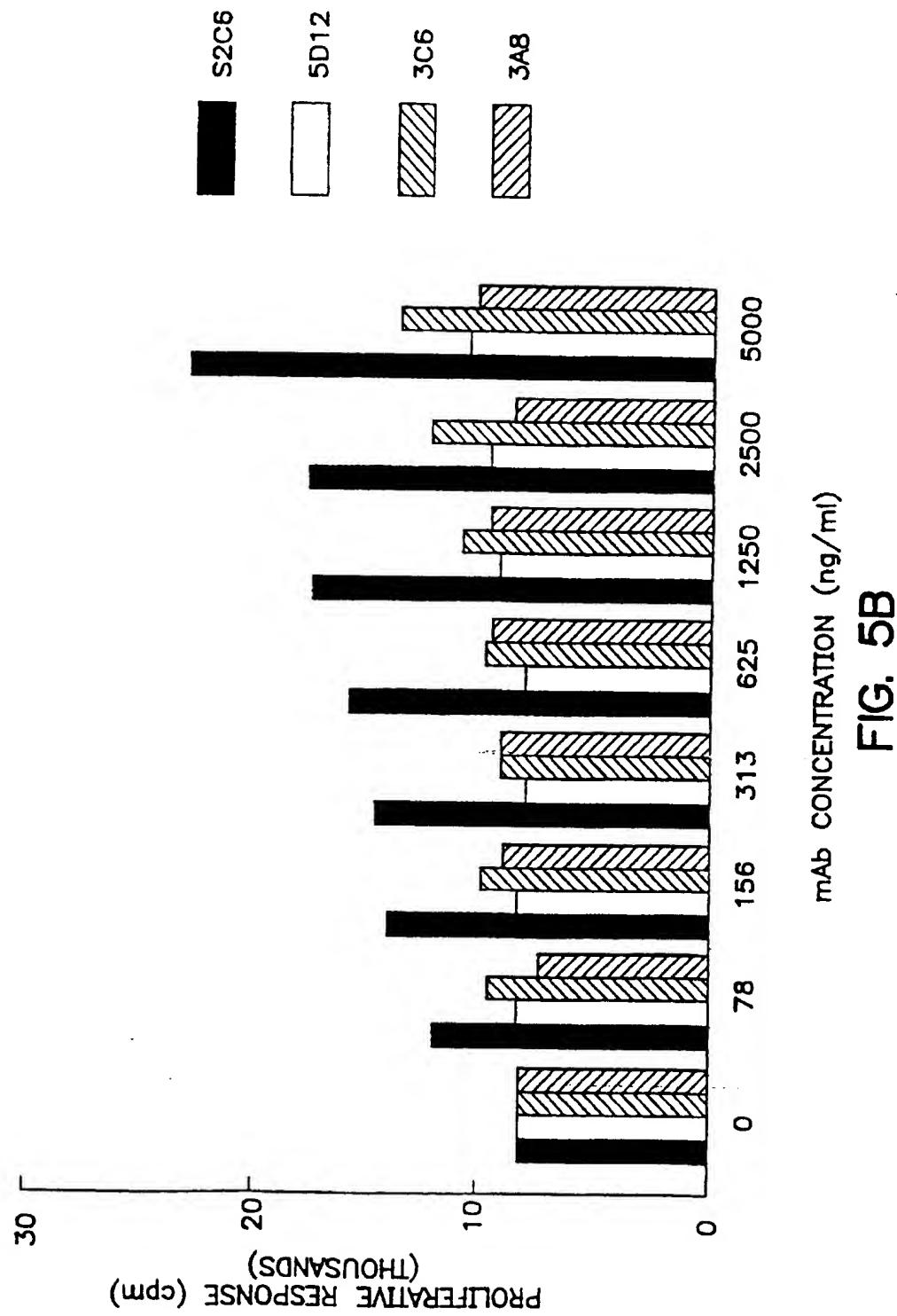


FIG. 4A





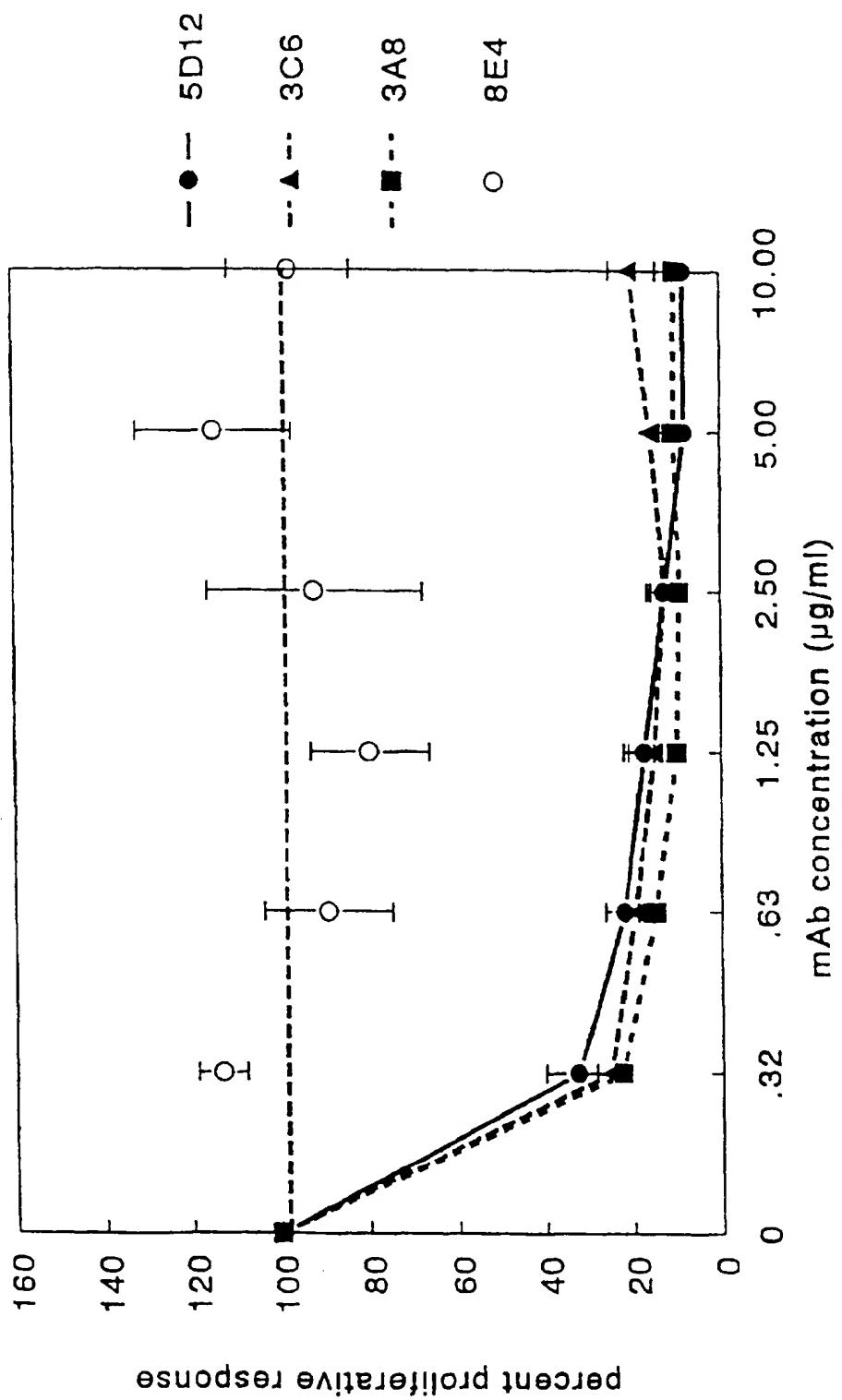


FIG. 6

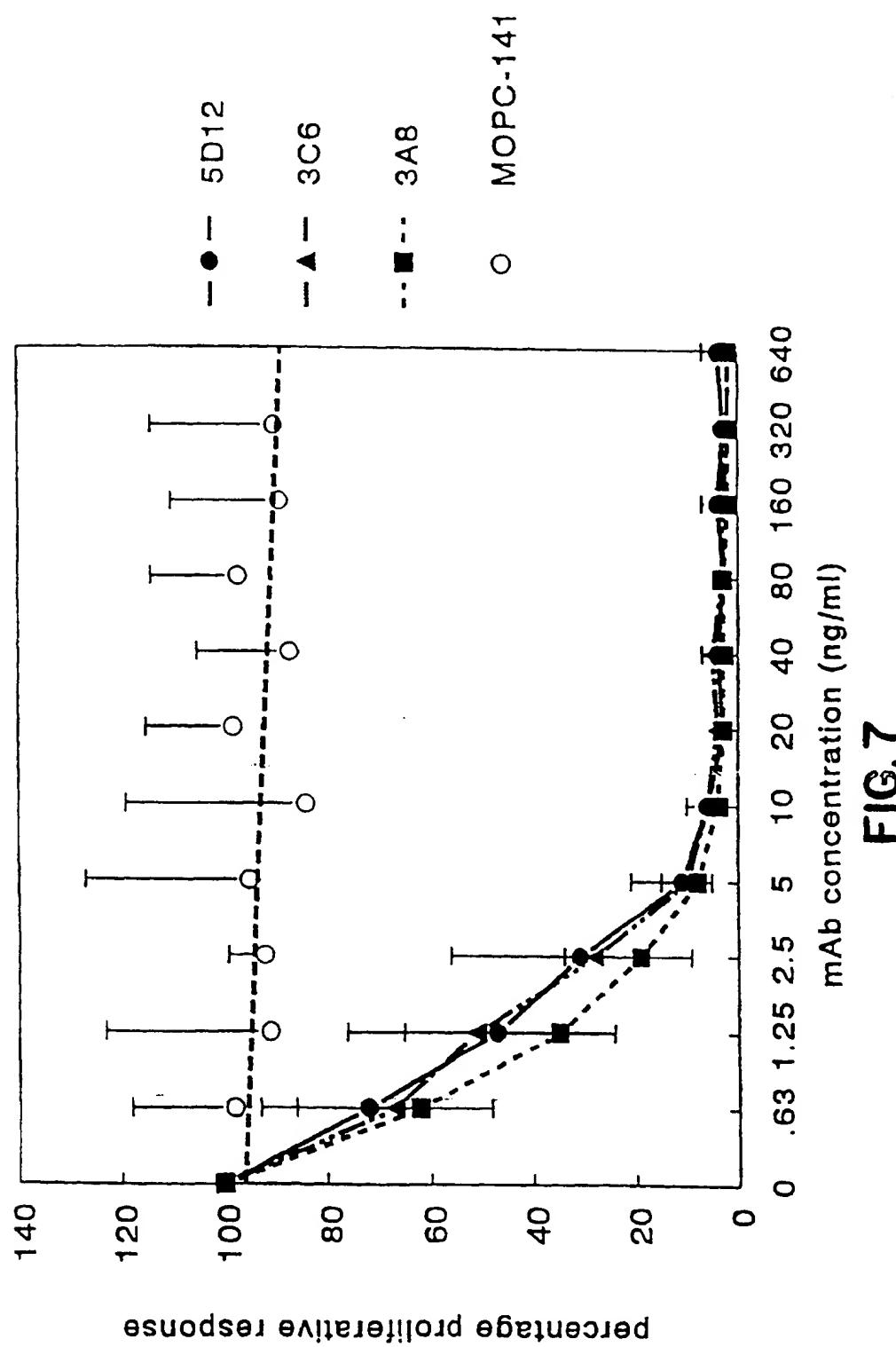


FIG. 7

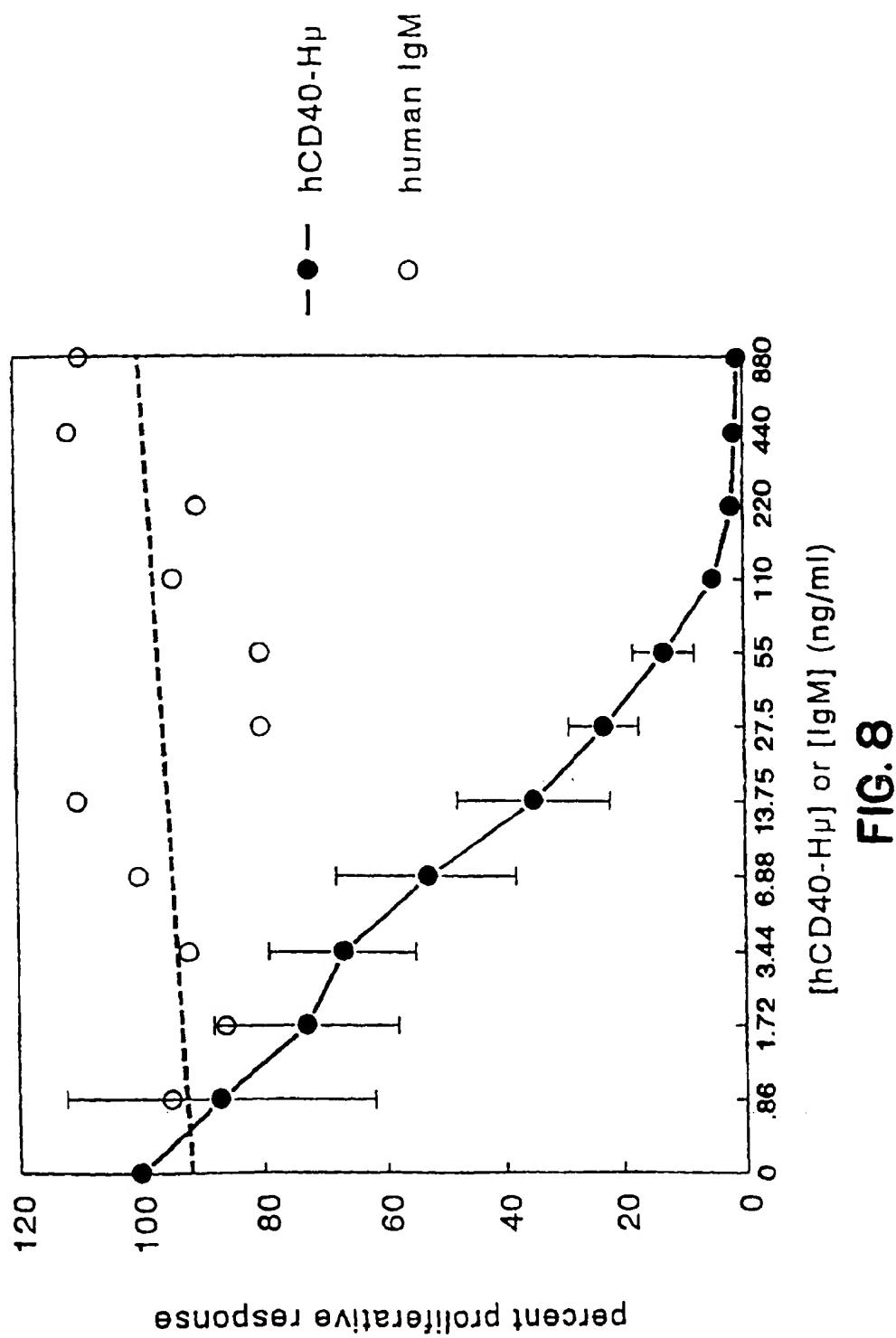


FIG. 8

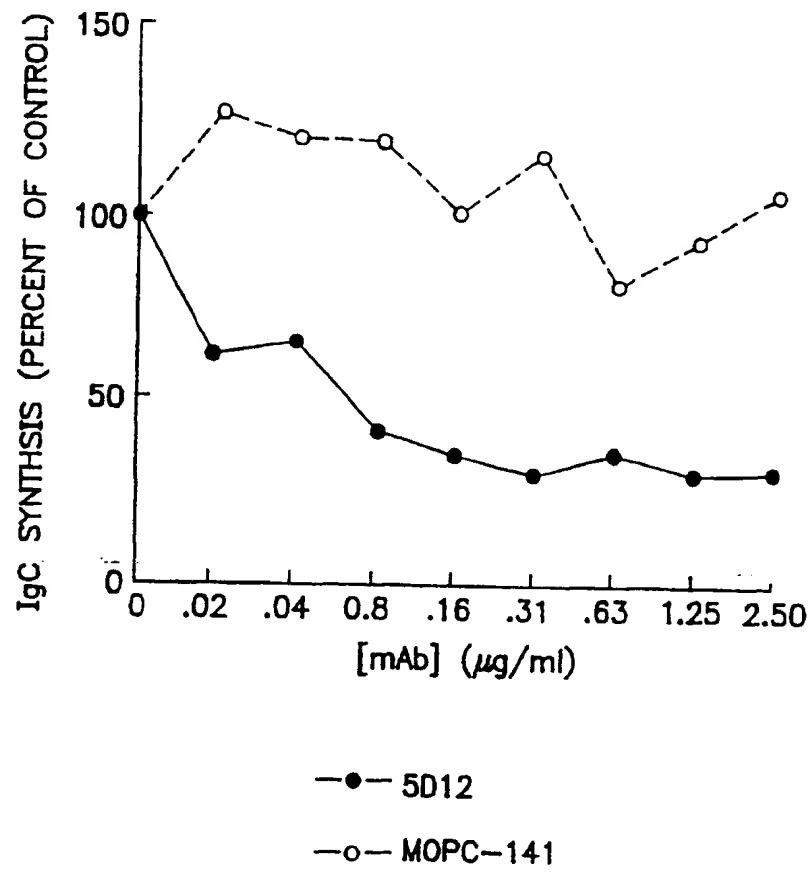


FIG. 9A

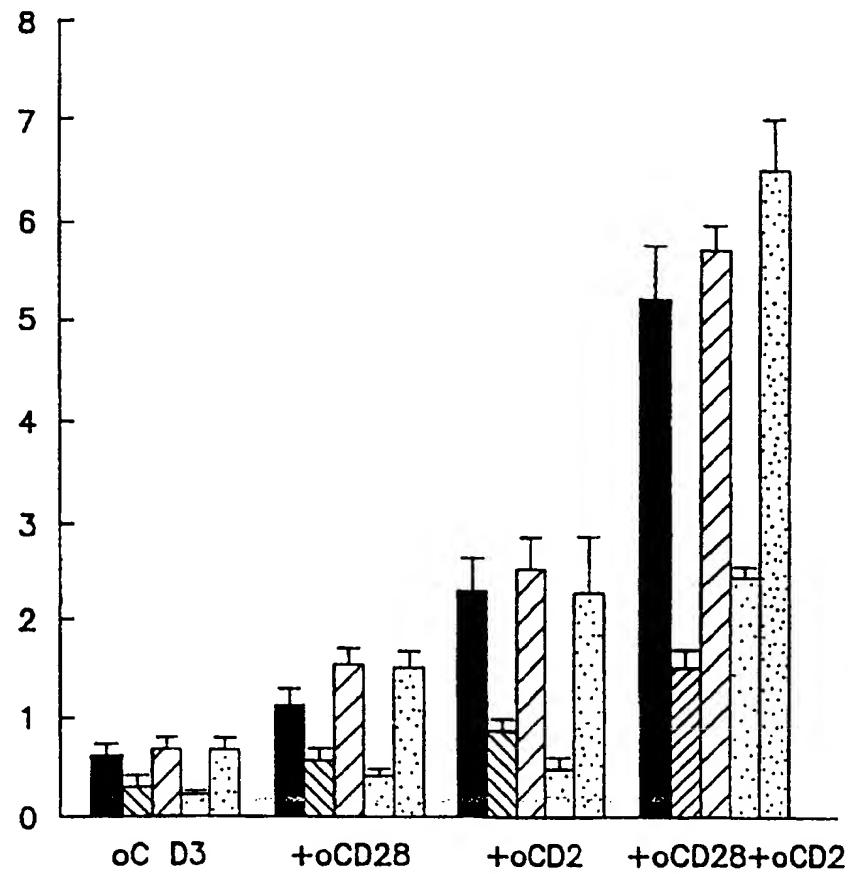


FIG. 9B

- NO ADDITION
- ▨ ANTI-CD40
- ▨ MOPC-141
- ▨ hCD40-H μ
- ▨ hIgM

1	FR1	CAG	GTC	AAG	CTC	GAG	TCT	GGA	CCT	GGC	CTG	GTC	GCA	CCC	TCA	CAG	48
	Q	V	K	L	E	S	G	P	G	L	V	A	P	S	Q	16	
49	AGC	CTG	TCC	ATC	ACA	TGC	ACT	GTC	ICT	GGG	TTC	TCA	TTA	TCC	AGA	TAT	96
17	S	L	S	T	C	T	V	S	G	F	S	L	S	R	Y	32	
97	AGT	GTA	TAC	FR2	TGG	GTT	CGC	CAG	CCT	CCA	GGA	AAG	GGT	CTG	GAG	TGG	144
33	S	V	Y	W	R	V	Q	P	P	G	K	G	L	E	W	L	48
145	GGA	ATG	ATG	TGG	GGT	GGT	GGA	TCC	ACA	GAC	TAT	AAT	TCA	GCT	CTC	AAA	192
49	G	M	M	W	G	G	G	S	T	D	Y	N	S	A	L	K	64
193	TCC	AGA	CTG	AGC	ATC	AGC	AAG	GAC	ACC	TCG	AAG	AGC	CAG	GTC	TTT	TTA	240
165	S	R	L	S	K	I	S	K	D	S	K	S	Q	V	F	L	280
241	AAA	ATG	AAC	AGT	CTG	CAA	ACT	GAT	GAC	ACA	GCC	ATG	TAC	TGT	GTC	288	
81	K	M	N	S	L	Q	T	D	D	T	A	M	Y	Y	C	Y	296
289	AGA	ACC	GAT	GGG	GAC	TAC	FR4	TGG	GGT	GAA	GGA	ACC	TCA	GTC	TCC	336	
97	R	T	D	G	D	Y	W	G	Q	G	T	S	V	T	S	112	
337	TCA	S														339	
113																113	

FIG. 10

1	GAG	CTC	CAG	CTG	ACC	CAG	TCT	CCA	CTC	TCC	CTG	CCT	GTC	AGT	CTT	GGA	48
	E	L	Q	L	T	Q	S	L	P	V	S	L	P	V	S	L	6
	FR1																16
49	GAT	CAA	GCC	TCC	ATC	TCT	TGC	AGA	TCT	AGT	CAG	AGC	CTT	GTA	AAC	AGT	96
	D	Q	A	S	I	S	C	R	S	Q	S	L	V	N	S	S	32
	CDR1																
97	AAT	GGA	AAC	ACC	TAT	TTA	CAT	TGG	TAC	CTG	CAG	AAG	CCA	GGC	CAG	CTC	144
	N	G	N	T	Y	L	H	W	Y	L	Q	K	P	G	Q	S	48
	CDR2																
145	CCA	AAG	CTC	CTG	ATC	TAC	AAA	GTT	TCC	AAC	CGA	TTT	TCT	GGG	GTC	CCA	192
	P	K	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	64
	CDR3																
193	GAC	AGG	TTC	AGT	GGC	AGT	GGA	TCA	GGG	ACA	GAT	TTC	ACA	CTC	AAG	ATC	240
	D	R	F	S	G	S	G	S	T	D	F	T	L	K	I	I	80
241	AGC	AGA	GTC	GAG	GCT	GAG	GAT	CTG	GGA	GTT	TAT	TTC	TGC	TCT	CAA	AGT	288
	S	R	V	E	A	E	D	L	G	V	F	C	S	Q	S	S	96
	CDR3																
289	ACA	CAT	GTT	CCG	TGG	ACG	GGT	GGA	GGA	GGC	ACC	AAG	CTG	GAA	ATC	AAA	336
	T	H	V	P	W	T	F	G	G	G	T	E	L	E	I	K	112

FIG. 11

1	FR1	GAG	CAA	CTC	GTG	GAG	TCT	GGG	CCT	GGC	CTG	GTG	AAA	CCC	TCA	CAG	48	
	Q	V	Q	L	V	E	S	G	P	6	V	K	P	S	Q	16		
49	AGC	CTG	TCC	ATC	ACA	TGC	ACT	GTC	TCT	GGG	TTC	TCA	TTA	TCC	AGA	TAT	96	
	S	L	S	1	T	C	T	V	S	G	F	S	L	S	R	Y	32	
17																		
97	AGT	GTA	TAC	FR2	TGG	GTT	CGC	GAG	CCT	CCA	GGG	AAG	GGT	CTG	GAG	TGG	CTG	144
	S	Y	W	Y	R	Q	P	G	P	G	K	G	L	E	W	L	48	
33																		
145	GGA	ATG	TGG	GGT	GGT	GGA	TCC	ACA	GAC	TAT	AAT	TCA	GCT	CTC	AAA	192		
	G	M	W	G	G	G	S	T	D	Y	N	S	A	L	K	64		
49																		
193	TCC	AGA	CTG	ACC	ATC	AGC	AAG	GAC	ACC	TCG	AAG	AAC	CAG	GTC	TTG	TTA	240	
	S	R	R	T	T	I	S	K	T	S	K	N	Q	V	F	L	80	
65																		
241	AAA	ATG	AAC	AGT	CTG	CGA	GCT	GAG	GAC	ACA	GCC	ATG	TAC	TGT	GTC	288		
	K	M	N	S	L	R	A	E	D	T	A	M	Y	C	V	96		
81																		
289	AGA	ACC	GAT	GGG	GAC	TAC	FR4											
	R	T	D	G	D	Y	W											
297																		
337	TCA																	
113	S																	

FIG. 12

1	GAC	CTC	CAG	CTG	ACC	CAG	TCT	CCA	CTC	TCC	CTG	CCT	GTC	AGT	CCT	GGA	48	
1	D	Q	L	Q	T	Q	S	P	L	S	P	Y	S	L	G	16		
49	GAT	CGA	GCC	TCC	ATC	TCT	TGC	AGA	TCT	AGT	CAG	AGC	CCT	GTA	AAC	AGT	96	
17	D	R	A	S	I	S	C	R	S	S	Q	S	L	V	N	S	32	
97	AAT	GGG	AAC	ACC	TAT	TTA	CAT	TGG	TAC	CTG	CAG	AAG	CCA	GGC	CAG	TCT	144	
33	N	G	N	T	Y	L	H	W	Y	L	Q	K	P	G	Q	S	48	
145	CCA	AAG	CTG	CTG	ATC	TAC	AAA	GTT	TCC	AAC	CGA	TCT	TTT	TCT	GGG	GTC	CCA	192
49	P	K	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	64	
193	GAC	AGG	TTC	AGT	GGC	AGT	GGG	TCA	GGG	ACA	GAT	TTC	ACA	CTC	AAG	ATC	240	
65	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	80	
241	AGC	AGA	GTG	GAG	GCT	GAG	GAT	GTG	GGG	GTT	TAT	TAC	TGC	TCT	CAA	AGT	288	
81	S	R	V	E	A	E	D	V	G	Y	C	Y	C	S	Q	S	96	
289	ACA	CAT	GTT	CCG	TGG	ACG	FR4	GGT	GGG	ACC	AAG	CTG	GAA	ATC	AAA	336		
97	T	H	Y	P	W	T	F	G	G	T	K	E	1	K	1	112		

FIG. 13

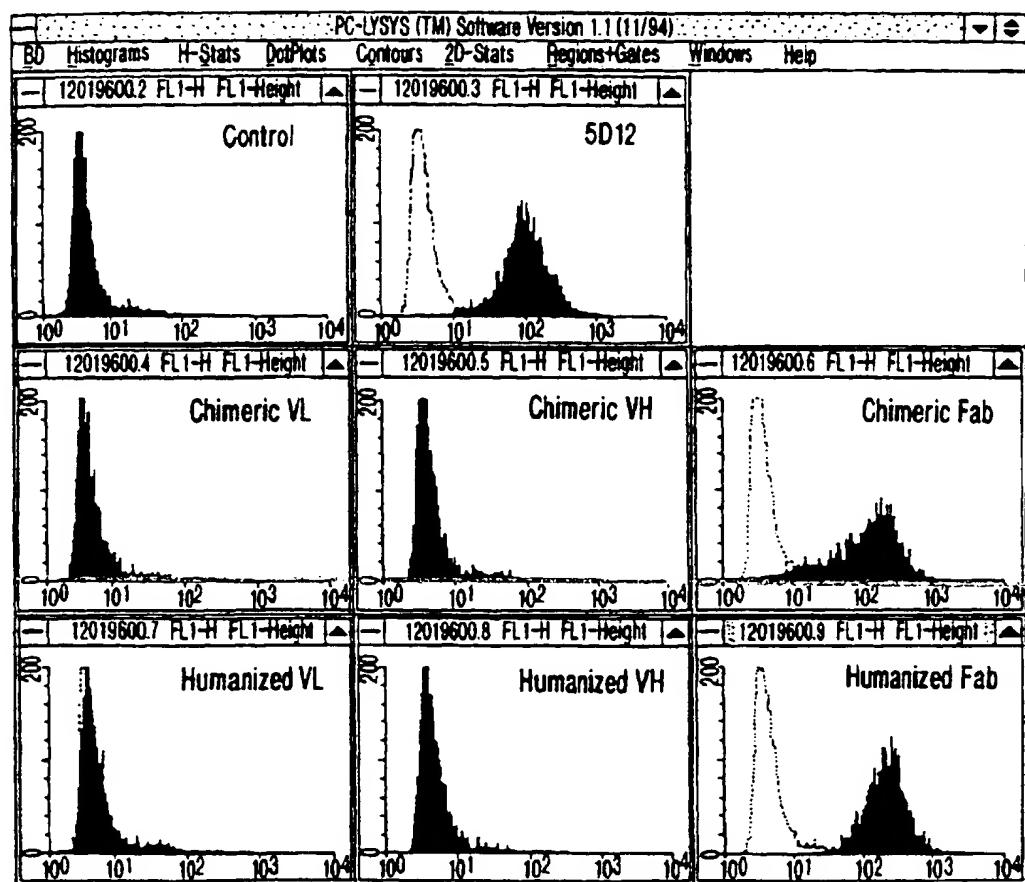


FIG. 14

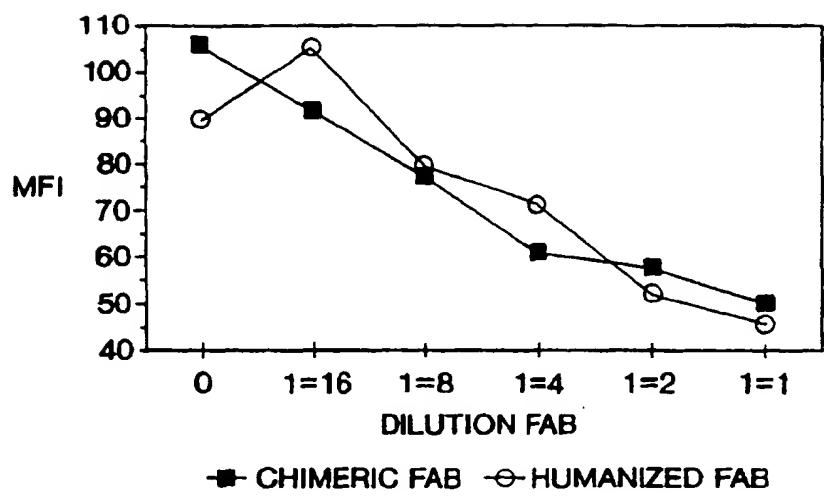


FIG. 15